ABSTRACT

Title of Document: BIOFILM FORMATION BY *ESCHERICHIA COLI* O157:H7

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*Escherichia coli* O157:H7 from cattle was evaluated for its ability to produce biofilm on food contact surfaces and quorum sensing signals in various raw meat, raw poultry, and produce broths. Generally, the strain was able to attach and form the most biofilm on stainless steel. Transfer of cells attached to stainless steel was observed onto various raw meat, raw poultry, ready-to-eat deli meats, and produce products as high as $10^4$ CFU/cm$^2$. *E. coli* O157:H7 isolated from 14 animal, food, and human sources were characterized on antimicrobial susceptibility, ability to form biofilm, and production of curli fimbriae and cellulose. Strains isolated from cattle, retail chicken, and retail beef were able to form strong biofilms in addition to curli and cellulose production. Additionally, *E. coli* O157:H7 from retail chicken showed considerable antimicrobial resistance. This study suggests *E. coli* O157:H7 biofilms pose significant risk to continuous contamination of a variety of food products.

# BIOFILM FORMATION BY *ESCHERICHIA COLI* O157:H7

By

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# Chapter 1: Introduction

Foodborne illnesses have major social and economic impacts. Foodborne illnesses bear an annual cost of an estimated $6.5 to $13.3 billion (Buzby and Roberts 1998). In 1993, treatment costs for cases of acute *E. coli* O157:H7 disease was estimated between $29.4 and $59.9 million (Buzby and others 1996). Costs included physicians' visits, laboratory tests, hospital fees, and dialysis and medication for patients who develop hemolytic uremic syndrome (HUS). In addition to medical costs, foodborne illness causes economic burden to the food industry.

Product recalls due to pathogenic contamination cost the manufacturer financial losses. These losses include direct costs from the recalls as well as related claims and legal proceedings. In 1996, Odwalla apple juice was recalled due to contamination with *E. coli* O157:H7, ultimately costing the company 12.4% of sales in fiscal 1997 and 2.1% of sales in 1998 (Salin and Hooker 2001). In 2007, a recall of 21.7 million pounds of frozen hamburger patties contaminated with *E. coli* O157:H7 forced Topps Meat Co. out of business (Wall Street Journal Staff 2007). Initially, 332,000 pounds of frozen hamburger patties had been recalled, indicating the need for control of contamination from an economic perspective besides disease prevention.

Formation of biofilm, extracellular polysaccharides that shield the bacteria against harsh environment, can serve as a source of microbial contamination during

processing. This study evaluated ability of *E. coli* O157:H7 isolated from cattle to form biofilm on food contact surfaces in a variety of food broths. The ability of *E. coli* O157:H7 to form biofilm on food contact surfaces decreases the effectiveness of sanitation processes therefore increasing the risk of contamination. The ability of the biofilm forming strain bound to contact surfaces to be transferred to various food products was investigated. Fourteen strains of *E. coli* O157:H7 from various animal, human, and food sources were employed to assess their ability to form biofilm and to produce cellulose and curli fimbriae. These stains' antimicrobial resistance profiles were also characterized.

Microbial contamination is a liability for food processors. The ability to effectively control contamination will greatly benefit consumer food safety and the economic welfare of the manufacturer. Understanding the ability of *E. coli* O157:H7 to form biofilms on various surfaces that come into contact with food in different sectors of the food industry should provide some insights on how to prioritize or strategize processing conditions that enhance safeguarding of our food supplies. Therefore, the objectives of this study were: (1) to demonstrate the ability of *E. coli* O157:H7 to form biofilm on food contact surfaces and cause contamination of food products, and (2) to characterize the ability of *E. coli* O157:H7 isolated from various sources to form biofilms.

# Chapter 2: Literature Review

## *2.1 Escherichia coli O157:H7*

### 2.1.1 *Escherichia coli* defined

*Escherichia coli* is a group of Gram-negative rod-shaped facultative anaerobes that are motile by peritrichous flagella (Thakur 2007). *E. coli* species are commonly classified by their virulence properties, mechanisms of pathogenicity, clinical syndromes, and O and H serotype (Montville and Matthews 2005). *E. coli* O157:H7 is classified by its serotype, O157:H7, because it expresses the 157$^{th}$ somatic (O) antigen and the 7$^{th}$ flagellar (H) antigen (Mead and Griffin 1998). Of diarrheagenic strains, *E. coli* O157:H7 is the predominant serotype of enterohemorrhagic *E. coli* (EHEC). Other categories of diarrheagenic *E. coli* are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC), and enteroaggregative *E. coli* (EAEC) (Montville and Matthews 2005).

EPEC organisms are classified as such because they have the ability to adhere to cells and produce lesions, as well as invade epithelial cells (Montville and Matthews 2005). However, EPEC species do not have the ability to produce toxins, unlike ETEC. ETEC organisms produce both heat liable and heat stable toxins, similar to those produced by *Shigella*. The method of pathogenicity of ETEC is to first colonize the small intestine, followed by toxin production (Montville and

Matthews 2005). ETEC is the major cause of traveler's diarrhea and disease in children (Thakur 2007). Unlike ETEC, EIEC is able to penetrate the cells of the large intestine (Thakur, 2007). The method of pathogenicity of EIEC is to first invade the epithelial cells and then multiply within them (Montville and Matthews 2005). Rather than penetrating the cell membrane, DAEC strains adhere in a random fashion to HEp-2 or HeLa cell lines, and may or may not produce Shiga toxins (Montville and Matthews 2005). DAEC infections are most common in children between the ages of 1 and 5, however, it is unknown as to why (Montville and Matthews 2005). EAEC is classified due to the nature they adhere to HEp-2 cell lines. EAEC strains are so named because they cluster and stack on the surface of cells (Montville and Matthews 2005). Finally, EHEC strains produce Shiga toxins (Stxs) that are cytotoxic to Vero cells. However, many *E. coli* strains produce Stxs, therefore EHEC strains are classified on the ability to cause bloody diarrhea in humans (Montville and Matthews 2005). Serotype O157:H7 is the most common EHEC strain (Montville and Matthews 2005) and is recognized as a common bacterial cause of bloody and nonbloody diarrhea, causing an estimated 20,000 infections per year (Besser and others 1999).

2.1.2. Pathogenicity

*E. coli* O157:H7 is an important causative agent of severe gastrointestinal disease in humans, including hemorrhagic colitis. The pathogen causes the majority of sporadic and multiperson outbreaks of bloody diarrhea in the U.S. (Dean-Nystrom and others 2003). *E. coli* O157:H7 is also responsible for the majority of cases of hemolytic uremic syndrome (HUS), a major cause of acute renal failure in children

(Boyce and others 1995; Murinda and others 2004). Five to fifteen percent of children infected with *E. coli* O157:H7 will develop HUS (Besser and others 1999; Tarr and others 2005). Age is a risk factor for the development of HUS, other risk factors may include: female gender, absent or weak P1 antigen expression by red blood cells, raised white blood cell count, presence of bloody diarrhea, fever, and administration of antimotility agents or antimicrobials (Besser and others 1999; Tarr and others 2005). An estimated 5% of all *E. coli* O157:H7 cases will develop HUS, the remaining 95% of cases will spontaneously resolve within 5 to 7 days of the onset of bloody diarrhea, which typically occurs 1 to 2 days after the onset of abdominal cramps and nonbloody diarrhea which begin 3 to 4 days after ingestion (Figure 1-1; Mead and Griffin 1998). The mechanism by which *E. coli* O157:H7 causes HUS is unclear; however, it is believed that the organisms adhere closely to the mucosal cells of the large intestine, disrupting the brush border (Mead and Griffin 1998). This may be enough to cause non-bloody diarrhea. Damage to epithelial cells provides entry for Shiga toxins, lipopolysaccharides (LPS), and other inflammatory mediators into circulation. Toxins may then bind to the endothelial cells of kidneys and initiate HUS (Besser and others 1999). Shiga toxins in the kidneys may damage the endothelial cells, causing platelet and fibrin deposition, resulting in renal failure (Mead and Griffin 1998). The infectious dose of *E. coli* O157:H7 is as low as 10 to 100 cells (Feng and Weagant 2002).

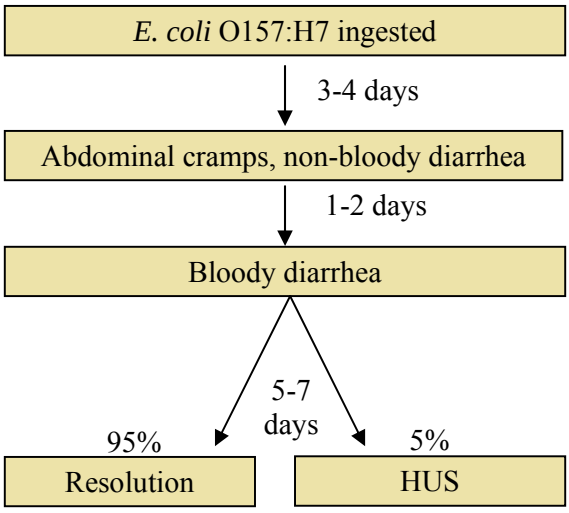



**Figure 2-1. Natural history of infection with *E. coli* O157:H7 infection. (adapted from Mead and Griffin 1998).** Symptoms of *E. coli* O157:H7 infections occur 3 to 6 days after infection. Most cases of *E. coli* O157:H7 are self limiting, however, about 5% of cases develop into hemolytic uremic syndrome.

2.1.3. Foodborne infection

The most common mode of transmission of *E. coli* O157:H7 infection is through consumption of contaminated food and water, although person to person infection is also possible (Besser and others 1999). *E. coli* O157:H7 naturally colonizes the intestinal tract of about 1% of healthy cattle, with highest numbers in the large intestine, and is shed by cattle from $10^2$ to $10^5$ CFU/g of feces (Boyce and others 1995; Moxley 2004; Campbell and others 2001). Foodborne infection by the pathogen was first observed in 1982 and associated with the consumption of contaminated ground beef (Besser and others 1999; Meng and others 1998a). Beef may become contaminated during slaughter, and the process of grinding can incorporate the pathogen to the inside of the product making thorough cooking necessary to eliminate the bacteria (Besser and others 1999). McGee and others

(2002) demonstrated the transmission of the pathogen from contaminated cattle to farm water and soil through fecal contamination. Although ground beef is commonly associated with *E. coli* O157:H7 infections, the number of produce-related cases is on the rise (Mead and Griffin 1998).

Produce associated outbreaks of *E. coli* O157:H7 were first reported in 1991, and accounted for 21% of foodborne outbreaks and 34% of foodborne outbreak-related cases from 1982 to 2002 (Rangel and others 2005). There has been an increase in *E. coli* O157:H7 outbreaks associated with produce products, such as lettuce, apple cider, cantaloupe, and alfalfa sprouts in recent years (Sivapalasingam and others 2004). In 2006 a multi-state outbreak of *E. coli* O157:H7, resulting in 199 illnesses and three deaths, was attributed to contaminated spinach (Centers for Disease Control and Prevention, CDC, 2006a).

## *2.2 Bacterial Biofilms*

### 2.2.1 Bacterial biofilms defined

Bacteria have the ability to attach, colonize, and form biofilms on a variety of surfaces (Uhlich and others 2006). Donlan and Costerton (2002) defined bacterial biofilms as a microbial community of cells that are irreversibly attached to a biotic or abiotic surface, or each other, embedded in a matrix of extracellular polymeric substances produced by the bacteria, which exhibit an altered phenotype in respect to growth rate and gene transcription. Biofilms are formed in distinct steps: (i.) initial reversible attachment of bacteria to a surface, (ii.) production of exopolysaccharide (EPS) material to form an extracellular polymeric matrix resulting in irreversible attachment, (iii.) early development of biofilm architecture, and finally (iv.)

maturation (Figure 1-2) (Chmielewski and Frank 2003; Kim and Wei 2007). The formation of biofilms increase bacterial resistance to environmental stresses such as, nutritional and oxidative stresses, desiccation, UV light exposure, sanitizing agents and anitmicrobials (Fatemi and Frank 1999; Costerton and others 1999).

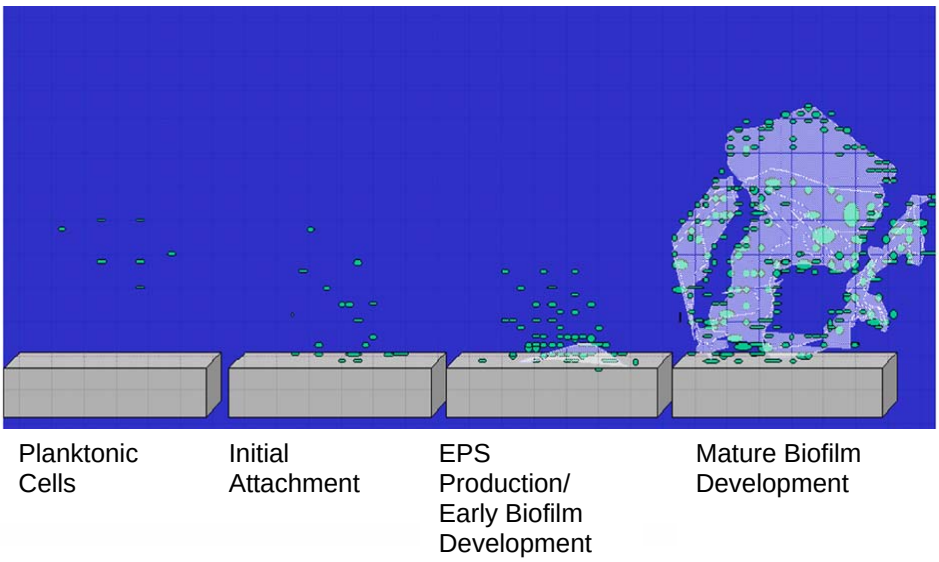



**Illustration 2-1. The process of biofilm formation (adapted from Chmielewski and Frank 2003).** Biofilm formation begins with initial reversible attachment of planktonic cells to a surface. Exopolysaccharide production and early biofilm development make attachment of bacteria to the surface irreversible. Finally, the biofilm develops into a complex matrix.

It has been reported that biofilm formation increases resistance to antimicrobial agents through a number of mechanisms. The most common theory is that the polymeric substances forming the matrix create a physical barrier, therefore decreasing penetration of the antimicrobial (Costerton and others 1999). A second theory of increased antimicrobial resistance due to biofilm formation is that some bacteria within the biofilm experience nutrient limitation and enter a slow-growing or starved state, which subsequently decreases the effectiveness of the antimicrobial

being administered (Costerton and others 1999). Since one biofilm community contains cells in varying metabolic states, it is likely that at least some bacteria will survive and attack (Costerton and others 1999). Due to this increased resistance to stresses biofilms pose a threat in both the food industry as well as clinical setting. From a medical perspective, biofilm formation on implanted medical devices such as catheters, prosthetic heart valves, joint prostheses, cardiac pacemakers, and shunts can be the source of persistent infection, leading to the characterization of a new infectious disease called chronic polymer-associated infection (Hall-Stoodley and others 2004).

### 2.2.2 Biofilms in the food industry

In addition to increasing resistance to sanitation, biofilm formation in food processing facilities increases opportunities of contamination of the processed product (Chmielewski and Frank 2003). Improperly cleaned and sanitized equipment is a major source of contamination. Cleaning-in-place (CIP) procedures, if not designed and implemented correctly, may not be able to effectively remove microbes accumulated on equipment surfaces, resulting in biofilm formation (Kumar and Anand 1998). Other common sites of biofilm accumulation are floors, waste water pipes, and bends in pipes, rubber seals, conveyer belts, and stainless steel surfaces (Kumar and Anand 1998).

Rodríguez and McLandsborough (2007) demonstrated the ability of *Listeria monocytogenes* attached to and in biofilms on the surface of stainless steel and high-density polyethylene to be transferred to ready-to-eat (RTE) bologna and American cheese. Furthermore, the *L. monocytogenes* existing within a biofilm community was

transferred to both RTE products with greater efficiency than the attached bacteria not within a biofilm (Rodríguez and McLandsborough 2007). Midelet and Carpentier (2002) observed greater transfer of *L. monocytogenes*, *Comamonas* spp., *S. sciuri*, and *P. putida* in biofilms on the surface of the polymers polyurethane and polyvinyl chloride to beef than those in biofilms on the surface of stainless steel.

The ability of bacteria to form biofilms on biotic surfaces poses a particular threat to food processors of minimally processed produce products. Plants are able to support bacterial growth due to their nutrient availability, water activity, and neutral pH, making produce a natural host for bacteria. *E. coli* O157:H7 biofilm formation has been observed on spinach, lettuce, Chinese cabbage, celery, leeks, basil, and parsley (Morris and others 1997; Pawar and others 2005). Biofilms naturally forming on plant surfaces are complex and include both Gram-negative and Gram-positive bacteria, yeasts and fungi (Charmichael and others 1999). A diverse biofilm increases the difficulty of removal and prevention of reemergence of pathogens and spoilage bacteria. Charmichael and others (1999) demonstrated a microbial load of $10^5$ CFU/g of predominately pseudomonads on lettuce could be reduced 100-fold with washing and sanitizing, however, after 9 days of storage bacterial counts were higher than before processing.

Not only do biofilms pose a particular risk for contamination in the food industry, but the development of biofilms may interfere with different processes. The formation of biofilms in potable water systems may clog pipes, decreasing velocity and carrying capacity, resulting in increased energy utilization (Kumar and Anand 1998). Biofilm formation in heat exchangers and cooling towers may also reduce

heat transfer and efficiency (Kumar and Anand 1998). The ability of bacteria persist in biofilms formed on metal surfaces in a processing facility could cause corrosion of the surface due to acid-production by the bacteria (Kumar and Anand 1998). Effective cleaning procedures are necessary for the prevention of dangerous and costly damage bacterial biofilms can cause.

An ideal cleaning and sanitation procedure should prevent bacterial accumulation and subsequent biofilm formation rather than focus on biofilm removal (Kumar and Anand 1998). Equipment design can be an important factor in controlling biofilm formation. Equipment should be designed to reduce bacterial accumulation and foster ease of cleaning (Chmielewski and Frank 2003). LeCercq-Perat and Lalande (1994) demonstrated no significant difference in cleanability of surface materials such as stainless steel, glass, nylon, and polyvinyl compounds when the materials were new, however, with time stainless steel better resisted damage caused by potentially harsh cleaning processes. However, implementation of procedures to remove biofilms is important to food industry as well.

There are a variety of physical methods being studied in the removal of biofilms from surface materials common in food processing facilities. Traditional strategy is mechanical removal, such as brushing (Kumar and Anand 1998). However, this may not be a feasible option for hard to reach areas. Newer physical methods of biofilm removal include super-high magnetic fields, ultrasound treatment, high pulsed electrical fields, as well as high pulsed electrical fields used in conjunction with organic acids, and low electrical fields on their own and in conjunction with biocides, such as silver, carbon, and platinum, and antibiotics

(Kumar and Anand 1998). A combination of cleaning and sanitizing is also used in food industry for the control and removal of biofilm.

An effective cleaning procedure would break up or dissolve EPS allowing sanitizers to gain access to viable bacteria (Chmielewski and Frank 2003). Alkaline cleaners, especially those with chelators like EDTA, were observed to be more effective at removing biofilms than acidic cleaners (Chmielewski and Frank 2003). In addition, superheated water was an effective cleaning method, although it was ineffective against 3 day old biofilm (Chmielewski and Frank 2003). Bacteria become far more susceptible to sanitizers once the biofilm matrix has been destroyed. Kumar and Anand (1998) note that oxidizing disinfectants such as peracetic acid, chlorine, iodine, and hydrogen peroxide, may depolymerize EPS enabling detachment of biofilms. Biological methods have also shown a certain level of success in the prevention and removal of biofilms. Nisin, an antimicrobial peptide approved for use in cheese spreads to control spores of *Clostridium botulinum*, has been shown to be successful in reducing attachment of *L. monocytogenes* to surfaces that has been treated with (Kumar and Anand 1998). Lastly, certain enzymes have been proven effective in disrupting EPS matrixes, thus allowing for the removal of biofilms (Kumar and Anand 1998). Understanding of biofilm formation is needed to develop effective means of prevention and treatment in order to reduce the risk of contamination. Attachment and subsequent biofilm formation may be influenced by a variety of physical and chemical factors of the cell including: certain bacterial properties such as relative negative charge of the cell (Dickson and Koohmaraie

1989), hydrophobicity of the cell, and the production of certain appendages such as fimbriae, curli, and other outer membrane proteins (Boyer and others 2007).

### 2.2.3 Role of curli production in formation of biofilm

Curli are long thin and wiry protein fibers on a cell's surface (Pawar and others 2005). Several curli-producing EHEC strains showed increased ability to bind to biotic and abiotic surfaces such as glass coverslips, polystyrene, and stainless steel (Boyer and others 2007). Furthermore, curli-producing EHEC strains have shown greater ability to attach to intestinal cells when compared to non-curli-producing strains (Boyer and others 2007). Curli may be expressed by certain *E. coli* strains under stressful environments as a means of survival (Boyer and others 2007). Bacteria under stress can employ a variety of mechanisms of survival; some of which may be coordinated through the phenomenon of quorum sensing, inter or intraspecies cellular communication. Furthermore, biofilm communities provide an optimal environment for cell-to-cell communication.

### 2.2.4 Role of quorum sensing in formation of biofilm

Quorum sensing is the ability of bacteria to regulate gene expression by communicating via autoinducer (AI) molecules (Pillai and others 2006). Gram-negative bacteria utilize acylhomoserine lactones (AHL) quorum signaling molecules also known as autoinducer-2 (AI-2) signals, while Gram-positive bacteria use short chain amino acids and peptides (Van Houdt and others 2004). There have been conflicting results on the relationship between quorum sensing and biofilm production. Several studies have linked quorum sensing to biofilm formation:

autoinducer-2 signals have been observed to control EPS production in *Vibrio cholerae*; homoserine lactones have been observed to control cell aggregation in *Serratia liquefaciens*; and a peptide signal produced by *Streptococcus mutans* has been observed to influence gene regulation in dental plaque (González Barrios and others 2006). Van Houdt and others (2004) observed no correlation between biofilm forming capacity and the production of quorum signals; however, they did recognize that production of quorum signals may trigger other biofilm related responses such as increased resistance to antimicrobials.

### 2.2.5 Objectives

The objectives of this study were to evaluate the ability of the *E. coli* O157:H7 strain isolated from cattle to form biofilm and produce quorum signals in various food substrates on food contact surfaces and the transfer of the bacteria attached to processing contact surfaces to the surface of food. Furthermore, *E. coli* O157:H7 from various isolation sources were characterized by their ability to form biofilm, produce curli and cellulose, and resist antimicrobials.

# Chapter 3: Production of biofilm and quorum sensing by *E. coli* O157:H7 and its transmission from contact surfaces to various food products

## *3.1 Introduction*

*Escherichia coli* O157:H7 is an important causative agent of severe gastrointestinal disease in humans. *E. coli* O157:H7 causes the majority of sporadic and multiperson outbreaks of bloody diarrhea in the U.S. (Dean-Nystrom and others 2003). *E. coli* O157:H7 infection is also responsible for most cases of hemolytic-uremic syndrome, a major cause of acute renal failure in children (Boyce and others 1995). The pathogen has a low infection dose, as low as 10 to 100 organisms (Feng and Weagant 2002). The Centers for Disease Control and Prevention (CDC) estimates that *E. coli* O157:H7 causes more than 70,000 illnesses and 60 deaths each year in the U.S. (Mead and others 1999).

*E. coli* O157:H7 can persist in the farm environment, soil, water, sediment, and animal carcasses (Mead and Griffin 1998). Healthy cows are known to be the major reservoir of *E. coli* O157:H7 (Barkocy-Gallagher and others 2001). The pathogen ranging from $10^2$ to $10^5$ CFU/g of feces is readily shed by cattle (Campbell and others 2001). McGee and others (2002) demonstrated the transmission of the pathogen from contaminated cattle to farm water and soil throughout the fecal contamination. A large number of outbreaks of *E. coli* O157:H7 have been associated with the consumption of contaminated ground beef and raw milk (CDC

1993). There has also been an increase in *E. coli* O157:H7 outbreaks associated with produce products, such as lettuce, apple cider, cantaloupe, and alfalfa sprouts in recent years (Sivapalasingam and others 2004). In 2006, multistate outbreaks of *E. coli* O157:H7 infections by the consumption of contaminated spinach and lettuce resulted in 199 illnesses in 26 states and 71 illnesses in 5 states, respectively (CDC 2006a; CDC 2006b).

*E. coli* O157:H7 has shown the ability to attach, colonize, and form biofilms on a variety of surfaces (Uhlich and others 2006). Bacterial attachment is the first step in contamination of foods, and it is enhanced by producing biofilms (Donlan and Costerton 2002). Biofilms are more resistant to environmental stresses, such as nutritional and oxidative stresses, desiccation, UV light exposure, and sanitizing agents (Fatemi and Frank 1999). Biofilms that are attached to food contact surfaces, such as stainless steel, polyvinyl chloride, and polyurethane, can serve as the continuous contamination source of food spoilage bacteria and pathogens in food processing environment (Mustapha and Liewen 1989; Midelet and Carpentier 2002).

*E. coli* O157:H7 is able to form biofilm not only on a variety of food processing surfaces but also on spinach, lettuce, Chinese cabbage, celery, leeks, basil, and parsley (Morris and others 1997; Pawar and others 2005). In addition, biofilm formation by *E. coli* O157:H7 can be attributed by autoinducer 2 (AI-2) signals that can be involved in the regulation of chemotaxis, flagellar synthesis, and motility genes (Pillai and Jesudhasan 2006; Gonzalez Barrios and others 2006). Production of biofilm and autoinducer 2 (AI-2) signals by *E. coli* O157:H7 can make it difficult to efficiently control cross-contamination of *E. coli* O157:H7 in food processing

environment. Therefore, the objectives of this study were to determine the effect of biofilm and quorum sensing production on the attachment of *E. coli* O157:H7 on food contact surfaces and to evaluate the transmission of the pathogen from food contact surfaces to various types of food products.

## *3.2 Materials and Methods*

### 3.2.1 Bacterial strains and culture broths

*E. coli* O157:H7 tested in this study was isolated from the cattle feces in a previous study (Kim and Wei 2007). The reporter strains (*Vibrio harveyi* BB170 and BB150) for autoinducer assay were kindly provided by Dr. Bassler of the Princeton University.

To prepare test broths for biofilm and autoinducer activity assays, raw ground beef, raw ground pork, raw ground chicken, raw ground turkey, cantaloupe, lettuce, alfalfa sprouts, and spinach were purchased from a local grocery store. For meat, poultry, and cantaloupe broths, each food sample (200 g) was homogenized with 800 ml of water, and then the samples were filter sterilized (0.2 μm; Nalgene, Rochester, N.Y.). For vegetable broths, each food sample (300 g) was blended with 600 ml of water and centrifuged (8,000 × g) at 4 °C for 10 min. Supernatants were then filter sterilized.

### 3.2.2 Biofilm assay

*E. coli* O157:H7 strain was overnight cultured at 37 °C in Luria-Bertani (LB) broth (Becton, Dickinson, Sparks, Md.). Biofilm-forming capability of the *E. coli* O157:H7 strain was determined by a crystal violet binding assay (Head and Yu 2004).

Overnight cell culture was diluted (1:100) in 0.1% peptone water (Becton, Dickinson). Ten µl of diluted culture was dispensed into wells of 96-well polyvinyl chloride (PVC) microplates (BD Biosciences, Bedford, Mass.) containing 90 µl of test broths. The plates were incubated for 6, 12, or 24 h at 25 °C. Bacterial growth was observed by measuring turbidity by reading absorbance at 595 nm with a microplate reader (Thermo Fisher scientific, Waltham, Mass.) Bacterial cultures were removed and centrifuged (17,900 × g) at 4 °C for 5 min. The supernatants were taken and stored at -20 °C for autoinducer activity assay. For biofilm assay, wells of the 96-well microplates were rinsed twice in deionized water to remove loosely attached bacteria. Each well was stained with 125 µl of 0.25% crystal violet for 30 min at room temperature. The staining solution was removed, and wells were rinsed in deionized water two times. After drying, the crystal violet bound to the biofilm was solubilized with 125 µl of 70% ethanol for 30 min. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, Calif.).

3.2.3 Autoinducer activity

The production of AI-2 by *E. coli* O157:H7 strain in meat, poultry, and vegetable broths was determined (Surette and Bassler 1998). The reporter strain, *Vibrio harveyi* BB170, was cultured overnight in autoinducer bioassay (AB) medium (0.3 M NaCl, 0.05 M $MgSO_4$, 0.2% Casamino Acids, 10 µM $KH_2PO_4$, 1 µM L-arginine, 20% glycerol) with shaking at 30°C. Overnight culture was diluted (1:5,000) in fresh AB medium. The diluted cells (90 µl each) were dispensed into each well of 96-well microplates (BD Biosciences). For autoinducer activity assay, each cell-free culture fluid of *E. coli* O157:H7 from various broths (10 µl) was added to the reporter

strain dispensed into 96-well microplates. Cell-free culture fluids of *V. harveyi* BB152 strain (AI-1$^-$, AI-2$^+$) and *E. coli* DH5α strain were included as a positive control and a negative control, respectively. The plates were incubated at 30°C for 3 h with shaking. Luminescence was measured by using a luminometer (Perkin-Elmer, Norwalk, Conn.).

3.2.4 Biofilm formation on surfaces of stainless steel and glass

The *E. coli* O157:H7 strain was overnight cultured in LB broth at 37 °C. The cultures were diluted (1:100) in 0.1% peptone water (Becton Dickinson) and inoculated into 200 ml of prepared beef, pork, chicken, turkey, cantaloupe, spinach, alfalfa sprouts, or lettuce broths at the final concentration of $10^5$ cells/ml. Aseptically, 8 ml of broths were dispensed into petri dishes (15 by 60 mm) containing stainless steel (20 by 20 mm, grade 304, finish no. 4) coupon or borosilicate glass (18 by 18 mm) chip. The petri dishes were incubated at 25 °C for 24 h. After incubation, the stainless steel or glass chip was transferred into a sterile petri dish and rinsed twice in 8 ml of deionized water. The surfaces of stainless steel coupons and glass chips were stained with 8 ml of 0.25% crystal violet for 30 min at room temperature. The staining solution was removed, and the chips were rinsed twice in 8 ml of deionized water. The crystal violet bound to the biofilm was solubilized with 5 ml of 70% ethanol for 30 min, and the absorbance was determined at 570 nm using a microplate reader (Bio-Rad).

### 3.2.5 Transmission of *E. coli* O157:H7 from surfaces of stainless steel to various types of foods

Transmission of *E. coli* O157:H7 from surfaces of stainless steel to beef, pork, chicken, turkey, ready-to-eat (RTE) salami, RTE ham, RTE turkey ham, cantaloupe flesh, lettuce, alfalfa sprouts, carrots, spinach, and green beans was enumerated using the pour plate method (Midelet and Carpentier 2002). Food samples that had been purchased in a local grocery store were cut into uniform size and shape; sprayed with 70% ethanol and stored in sterile bags (Whirl-Pak, Nasco Sampling Products, Ft. Atkinson, Wis.) at -20°C.

Overnight cell culture of the *E. coli* O157:H7 strain was inoculated into 500 ml of LB broth at the concentration of $10^5$ cells/ml and dispensed into petri dishes (15 by 60 mm) containing stainless steel (2 by 2 inch, grade 304, finish no. 4) coupons. The petri dishes were incubated at 28 °C for 3 h for attachment of *E. coli* O157:H7 on the surfaces. The stainless steel was transferred to sterile petri dishes and rinsed twice in sterile water on a rocking platform for 5 min to remove any loosely attached bacteria.

Each piece of food samples was placed into individual petri dishes (15 by 60 mm) and rinsed twice in sterile water on a rocking platform for 5 min. For enumeration of the transferred cells, individual pieces of food samples were placed on surfaces of stainless steel for 30 min at room temperature. The surface of the food sample in contact with the stainless steel was thoroughly swabbed with a sterile cotton swab moistened with 0.1% peptone water. To ensure ethanol efficiently sterilized the surface of food samples, samples not in contact with stainless steel were swabbed and enumerated as well. The swab was placed in a tube containing 5 ml of

0.1% peptone water and vortexed. Bacterial counts were determined in duplicate by the standard pour plate method (Food and Drug Administration 1992). Aseptically, one ml of broth was taken and mixed with 9 ml of peptone water (0.1%). Each sample was serially diluted, dispensed, and mixed with tryptic soy agar (Becton Dickinson). The plates were incubated at 37 °C for 24 h.

### 3.2.6 Statistical analysis

One-way analysis of variance (ANOVA) was used to determine variation in biofilm and AI-2 production in various types of broths and transmission of *E. coli* O157:H7 from surfaces of stainless steel to various food products. A significant difference was defined at $p<0.05$. All analyses were performed by using SPSS (SPSS 13.0.1 for Windows, SPSS Corporation, Chicago, Ill.).

## *3.3 Results and Discussion*

### 3.3.1 Production of biofilm and quorum sensing signals by *E. coli* O157:H7 in meat, poultry, and produce broths

In this study, the biofilm-forming capability of *E. coli* O157:H7 strain in beef, pork, chicken, turkey, cantaloupe, lettuce, alfalfa sprout, and spinach broths was evaluated (Figure 3-1B). There was little variation in the ability of the strain to grow in the different test broths at 24 h incubation (Figure 3-1A). The cantaloupe and spinach produce broths were able to support similar or greater amounts of growth of *E. coli* O157:H7 strain when compared with the meat (beef and pork) and poultry broths (chicken and turkey; $p<0.05$).

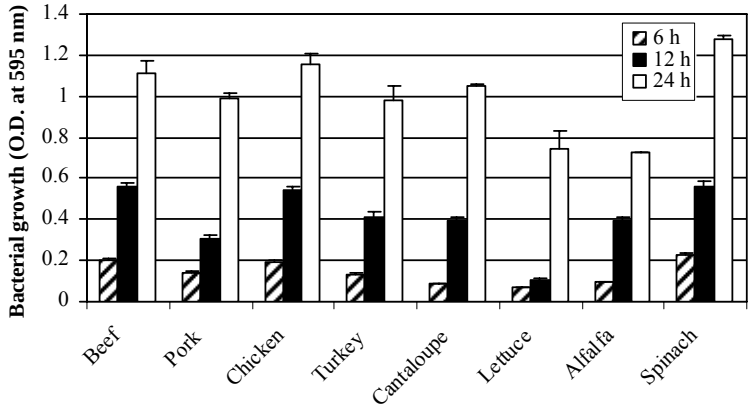
*E. coli* O157:H7 strain biofilms accumulate over time, therefore formation at 12 h was stronger than at 6 h, and biofilm formation at 24 h incubation was generally the strongest. There is no correlation between the strain's ability to form biofilms at 6 h and its ability to form biofilms at 24 h; although, broths which provide a good environment for biofilm formation by the *E. coli* O157:H7 strain at 12 h also support good formation at 24 h. At 12 h incubation, there was no significant difference in biofilm formation by the strain in the cantaloupe, alfalfa, and spinach broths and in formation of biofilm in all meat and poultry broths ($p<0.05$). Produce broths are assumed to have fewer nutrients than the meat and poultry broths. Our finding that produce broths support similar biofilm formation when compared to meat and poultry broths at 12 h disagrees with the findings of Dewanti and Wong (1995) that *E. coli* O157:H7 formed biofilms faster and with greater extracellular polysaccharide production when grown in medium with lower nutrient availability. However, it appears that biofilm formation is high in produce broths with 12 h incubation but formation begins to slow down with 24 h incubation. This agrees with the previous study that low nutrient broths foster an environment for faster biofilm formation (Dewanti and Wong 1995). Because biofilms can form quickly in produce broths regular sanitation is necessary to prevent the formation of biofilm on contact surfaces. It is possible that biofilm formation did not continue as strongly in produce broths when compared to meat and poultry broths because the nutrients of the produce broths were exhausted. This would not be the case in a produce processing facility where new substrates would be continually introduced into the environment. Pork broth supported the greatest amount of biofilm formation by the *E. coli* O157:H7

strain at 24 h, with levels significantly greater than beef, turkey, and all produce (cantaloupe, lettuce, alfalfa sprout, and spinach) broths ($p<0.05$). Although beef is classically associated with *E. coli* O157:H7 outbreaks, the strain did not form significantly greater biofilms in beef broth than in alfalfa and spinach broths at 24 h ($p<0.05$). This finding indicates that the increased incidence of *E. coli* O157:H7 infections from ground beef is more likely related to its reservoir in healthy cattle than the bacteria's ability to persist in biofilms in the processing environment. However, the strain is capable of forming biofilms in beef broth and may therefore increase difficulty of cleaning and sanitation practices in beef processing.

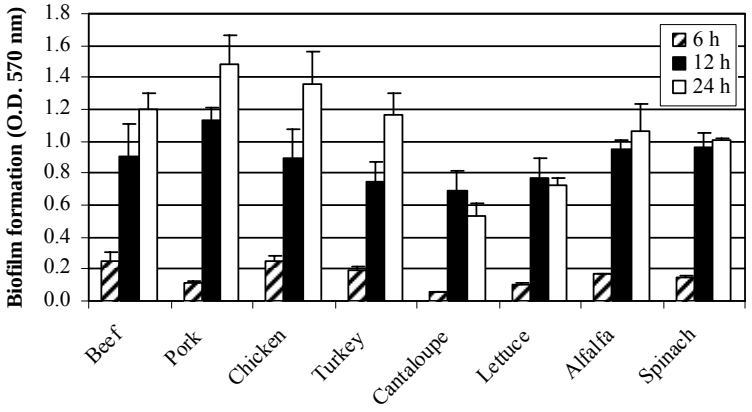
The ability of bacteria to form biofilms is dependent on many factors, one of which may be the production of quorum sensing signals, such as the AI-2 molecule. The *E. coli* O157:H7 strain typically produced the most AI-2 signals at 12 h (Figure 3-1C). This is true in pork and turkey broths where the strain's biofilm production was greatest at 24 h. It is possible that the ability of the cells to communicate via quorum sensing facilitated continuing biofilm production in pork and turkey broths. However, this trend is not present in spinach broth in which the *E. coli* O157:H7 strain produced the strongest AI-2 signal, but whose biofilm production did not continue from 12 h to 24 h. Our conflicting results are consistent with the literature on the relationship between quorum sensing and biofilm formation. González Barrios and others (2006) observed AI-2 signal molecules increased biofilm formation in *E. coli* by stimulating motility genes. Van Houdt and others (2004) found no correlation between the ability to form biofilms and AI-2 production in 26 gram negative isolates from a raw vegetable processing plant. Conflicting results suggest biofilm formation

is a complex process and quorum sensing may not be a factor although not an absolute determinant of biofilm formation.

A.




B.




C.

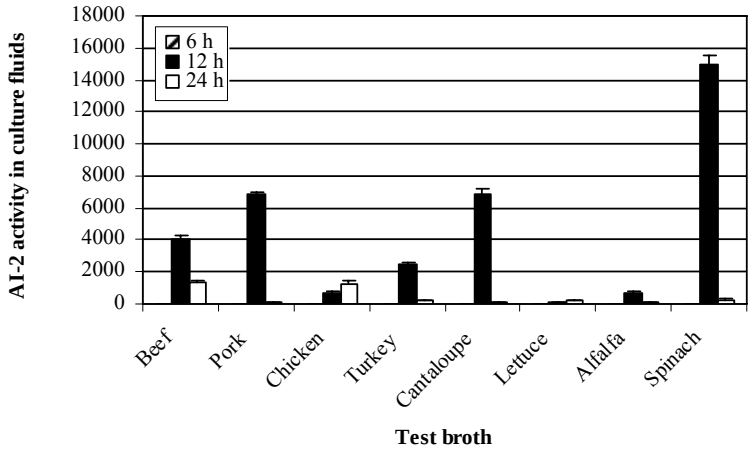



**Figure 3-1. Bacterial growth (A), biofilm formation (B), and AI-2 signal production (C).** Bacterial growth, biofilm formation, and AI-2 signal production of *E. coli* O157:H7 in the raw meat (beef and pork), raw poultry (chicken and turkey), and produce (cantaloupe, lettuce, alfalfa sprout, and spinach) broths was observed at incubation of 6, 12, and 24 h at 25 °C. Bacterial growth was observed by measuring turbidity at 595 nm. Determination of biofilm was done by crystal violet staining. AI-2 signal production was observed with the reporter strain *Vibrio harveyi* BB170 in an autoinducer bioassay. Error bars represent the standard deviation (n=3).

### 3.3.2 Biofilm formation on glass and stainless steel surfaces

In general, *E. coli* O157:H7 strain formed stronger biofilms on stainless steel than on glass, with the exception of cantaloupe broth (Figure 3-3). The ability of the strain to produce strong biofilms on glass in the cantaloupe broth was visually evident with crystal violet staining (Figure 3-2). Chicken broth supported significantly greater biofilm formation by the *E. coli* O157:H7 strain on stainless steel chips than in all other broths ($p<0.05$). The *E. coli* O157:H7 strain formed significantly greater biofilms in turkey and alfalfa sprout broths than in meat broths (beef and pork) and the other poultry broths on stainless steel ($p<0.05$). In a previous study, there was no significant difference in the ability of most isolates to form biofilms on stainless steel and glass (Kim and Wei 2007). However, this was not universally true and deviations were dependent on both strain and broth (Kim and Wei 2007).

*L. monocytogenes* forms greater biofilms on polymer surfaces used on conveyer belts in food processing facilities (polyvinyl chloride and polyurethane) than on stainless steel and subsequently have a greater potential for contaminating meat which came in contact with it (Midelet and Carpentier 2002). Although biofilms formed at greater levels on plastic-type materials than stainless steel, Rodríguez and McLandsborough (2007) demonstrated that stainless steel transferred more *L. monocytogenes* to food surfaces than high density polyethylene.

A.

i. ii. iii. iv.

B.

i. ii. iii. iv.

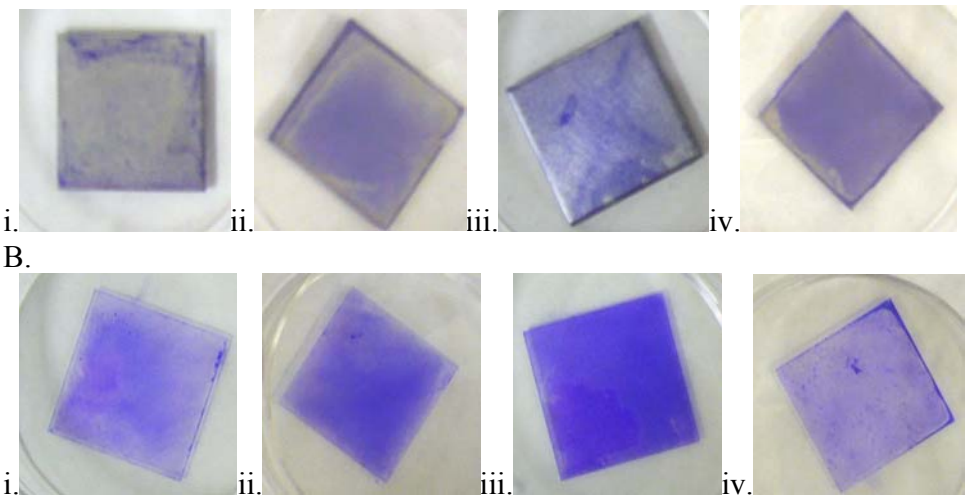


**Figure 3-2. Crystal violet staining of biofilm on glass and stainless steel.** Biofilm by *E. coli* O157:H7 strain was formed on stainless steel and glass coupons submerged in various raw meat (beef and pork), raw poultry (chicken and turkey), and produce (cantaloupe, lettuce, alfalfa sprout, and spinach) broths for 24 h at 25 ºC. Pictured is the biofilm stained with crystal violet on the surface of stainless steels (A) and glasses (B) in beef (i), turkey (ii), cantaloupe (iii), and alfalfa sprout (iv) broths.

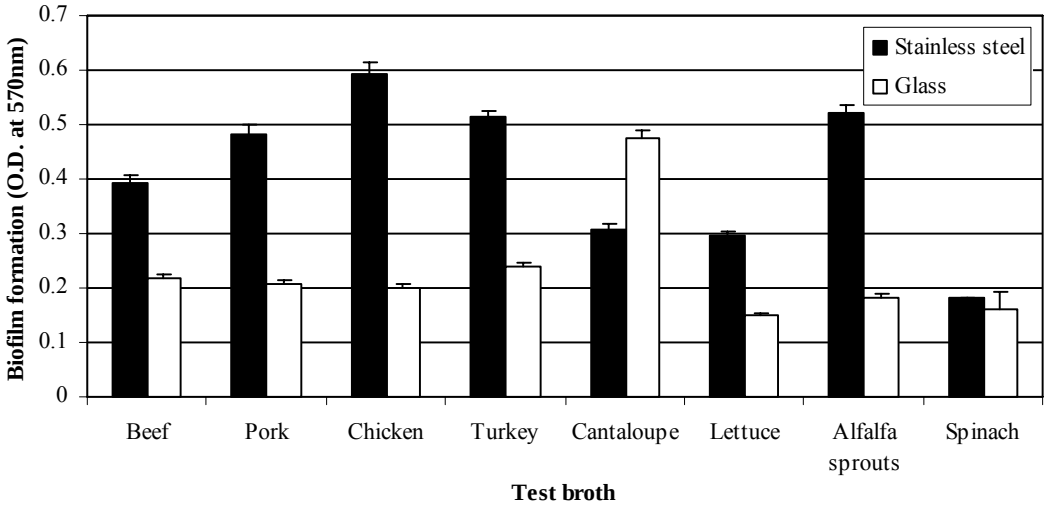



**Figure 3-3. Biofilm formation on stainless steel and glass.** Biofilm was formed by the *E. coli* O157:H7 strain on stainless steel and glass coupons submerged in various raw meat (beef and pork), raw poultry (chicken and turkey), and produce (cantaloupe, lettuce, alfalfa sprout, and spinach) broths for 24 h at 25 ºC. Determination of biofilm was done by crystal violet staining. Error bars represent the standard deviation (n=3).

### 3.3.3 Transfer of *E. coli* O157:H7 strain from stainless steel coupons to surface of meat, poultry, RTE deli, and produce products

Since *E. coli* O157:H7 strain can form strong biofilms in broth, we evaluated transmission of the bacteria attached to stainless steel coupons to food products (Figure 3-4). The foods tested can be categorized into four groups based on the amount of *E. coli* O157:H7 strain transferred from the stainless steel to the food sample. Cantaloupe is in a category of its own showing the greatest affinity for cross contamination, with only a 0.25 Log CFU/cm$^2$ reduction of *E. coli* O157:H7 strain transferred from stainless steel, significantly more bacterial transmission than all other food samples tested ($p<0.05$). This could be due to the porous nature of the cantaloupe flesh as well as the physical nature of the juice material from the cantaloupe itself. Raw pork and raw chicken make up the second highest group with similar loads of *E. coli* O157:H7, 4.41 ± 0.48 Log CFU/cm$^2$ being transferred from stainless steel to each food surface ($p< 0.05$). Raw turkey and RTE turkey ham comprise the third group with 0.56 and 0.71 Log CFU/cm$^2$ reductions in *E. coli* O157:H7 transferred from stainless steel to food surface, or transfer of 4.20 ± 0.30 Log CFU/cm$^2$ and 4.04 ± 0.20 Log CFU/cm$^2$ respectively, and therefore were significantly greater than the remaining tested foods ($p<0.05$). The remaining tested foods, raw beef, RTE salami, RTE ham, lettuce, carrot, spinach, alfalfa sprouts, and green bean transferred between 1.22 and 2.44 Log CFU/cm$^2$ of the strain attached to stainless steel to food surfaces.

In general, transmission of *E. coli* O157:H7 from stainless steel to produce products, with the exception of cantaloupe, was lower than any other one type of food. Interestingly, transmission of *E. coli* O157:H7 strain to raw beef was not significantly

greater than this group ($p<0.05$), even though raw beef is commonly associated with this bacteria. Our results on the cantaloupe and raw meats and poultry products, which have textured moist surfaces, agree with previous observations that the efficiency of transfer of bacteria from stainless steel to food products was not universally greater when the surface was dry or wet but dependent on both the food being tested and the water activity (Rodríguez and McLandsborough 2007). Furthermore, the muscle tissue of lean raw meats may be favorable for transmission and attachment of the *E. coli* O157:H7 strain.

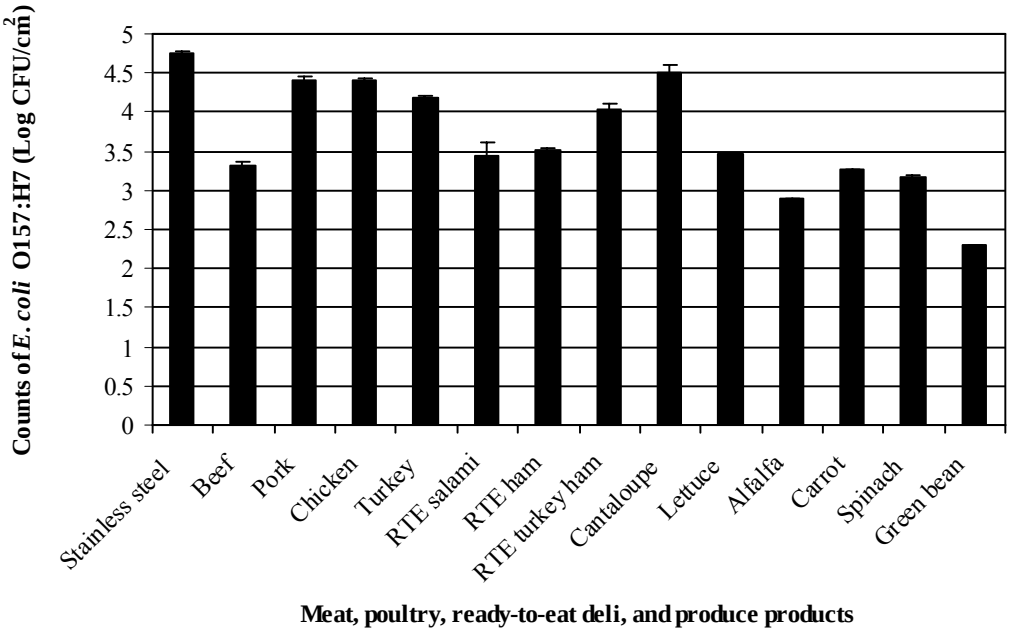



**Figure 3-4. Transfer of *E. coli* O157:H7 to various food materials.** *E. coli* O157:H7 strain was transferred from stainless steel coupons to various raw meat (beef and pork), raw poultry (chicken and turkey), RTE deli meats (salami, ham, and turkey ham), and produce products (cantaloupe, lettuce, alfalfa sprout, carrot, spinach, and green bean). Contact time was 30 min at room temperature. Counts of *E. coli* O157:H7 transferred are in terms of Log CFU/cm$^2$ where cm$^2$ is the area of the food material in contact with the stainless steel. Error bars represent the standard deviation (n=3).

It is important to note that all food samples tested contracted counts of *E. coli* O157:H7 on one square centimeter above the minimum infectious dose of 10 to 100 cells. The group with the least *E. coli* O157:H7 transferred, contracted between (2.0 ± 0.0) × $10^2$ CFU/$cm^2$ to green beans and (3.3 ± 0.1) × $10^3$ CFU/$cm^2$ to RTE ham. These products are of particular importance because no further processing to remove or destroy pathogens is necessarily done before they are consumed.

### *3.4 Conclusions*

The test broths were able to provide rich substrates for *E. coli* O157:H7 biofilm formation under conditions seen in food processing. These broths are similar to wash waters in food processing lines, indicating the rich environment capable of supporting biofilm formation by *E. coli* O157:H7. We also demonstrated the ability of *E. coli* O157:H7 attached to stainless steel to be transferred and attach to raw meat, raw poultry, RTE deli meats, and produce products.

# Chapter 4: Characterization of biofilm production of *E. coli* O157:H7 from various animal, human, and food sources

## *4.1 Introduction*

*Escherichia coli* O157:H7 is the predominant strain of Enterohemorrhagic *E. coli*. It causes the majority sporadic and multiperson outbreaks of bloody diarrhea in the U.S. (Dean-Nystrom and others 2003). Although most cases if *E. coli* O157:H7 infection result in self-limiting resolution, five to fifteen percent of cases in children develop into hemolytic uremic syndrome (HUS), a major cause of acute renal failure in children (Besser and others 1999; Tarr and others 2005; Boyce and others 1995). *E. coli* O157:H7 has shown the ability to attach, colonize, and form biofilms on a variety of biotic and abiotic surfaces (Uhlich and others 2006; Morris and others 1997; Pawar and others 2005).

Biofilms, a microbial community in a matrix of exopolysaccharide (EPS) material produced by the bacteria, enhance a bacteria's ability to survive (Donlan and Costerton 2002). The structure of biofilms increase resistance to a number of environmental stresses including nutritional and oxidative stress, UV light exposure, sanitizing agents, dehydration, and salinity (Fatemi and Frank 1999; Chmielewski and Frank 2003; Hall-Stoodley and others 2004). Improved survival may be attributed to inhibition of penetration of stresses or interactions between the biofilm and stress or interactions of an enzyme formed within the biofilm and a stress (Augustin and Ali-

Vehmas 2004). Biofilm formation also enhances antimicrobial resistance due to delayed penetration of the antimicrobial and an altered growth state within the biofilm structure (Donlan and Costerton, 2002). Antimicrobial resistance is of importance when treating infected patients although, the effectiveness of antibiotics in the treatment of *E. coli* O157:H7 and HUS is under continuing debate (Schroeder and others 2002; Phillips and others 2005). Pathogenic bacterial development of antimicrobial resistance is the result of clinical use of antibiotics. Resistance is also developed through the subtherapeutic use of antibiotics in animal feeds for disease prevention and growth promotion. These resistant strains are then introduced to humans through the food chain.

*E. coli* O157:H7 persists in a variety of animal, food, and clinical settings, and can be transferred from one environment to another making the origin of antimicrobial resistance difficult to locate. The interaction of bacteria within multiple environments has created strains with varying abilities to produce biofilm.

Adhesion and subsequent biofilm formation is effected by a number of physical and chemical properties of the cell (Kumar and Anand 1998; Chmielewski and Fank 2003). Curli are proteinaceous fibers of a complex extracellular matrix produced by many *Enterobacteriaceae* that aid in adhesion, aggregation, and biofilm formation (Barnhart and Chapman 2006). Cellulose was found to be a second component of the extracellular matrix with the ability to enhance biofilm formation and survival on surface environments (Zogaj and others 2001; Solomon and others 2005; Solano and others 2002).

Formations of biofilm on surfaces in food processing facilities are possible sources of contamination. Common sites of bacterial contamination include conveyors, collators, hand tools, gloves, and gaskets (Rodríguez and McLandsborough 2007). Materials such as stainless steel, polymeric materials (polyvinyl chloride, polyurethane, and high density polyethylene), glass, and rubber used for these purposes allow for bacterial colonization and biofilm formation (Rodríguez and McLandsborough 2007; Midelet and Carpentier 2002; Rivas and others 2007; Uhlich and others 2006; Kumar and Anand 1998).

The objectives of this study were to assess if curli and cellulose production enhance biofilm formation by *E. coli* O157:H7, and secondly, to evaluate if *E. coli* O157:H7 isolated from animal, human, and food sources form biofilm and to observe the ability of *E. coli* O157:H7 to form biofilm on food contact surfaces.

## *4.2 Materials and Methods*

### 4.2.1 Bacterial strains and culture broth

Fourteen isolated of *E. coli* O157:H7 were tested. Strains isolated from food, animal, and human sources (Table 4-1) were kindly provided by Dr. Jianghong Meng of the University of Maryland. Bacterial strains were stored in a Luria-Bertani (LB) broth (Becton Dickinson, Sparks, Md.) glycerol mixture (1:1 v/v) at -80 ºC until ready for use. They were grown overnight in LB broth. Broths were prepared from beef, pork, turkey, chicken, cantaloupe, lettuce, alfalfa sprout, and spinach as previously described. In addition, seafood broths were similarly prepared. Raw tilapia, cod, salmon, scallops, shrimp, and oysters were obtained from a local grocery

store. Each seafood sample (200 g) was blended with 800 ml of water, centrifuged (8,000 × g) at 4 °C for 10 min, and filter sterilized (0.2µm; Nalgene, Rochester, N.Y.).

4.2.2 Biofilm assay

The fourteen *E. coli* O157:H7 isolates were cultured overnight at 37 °C in LB broth. Turbidity to measure bacterial growth was observed by determining absorbance at 595 nm using a microplate reader (Thermo Fisher Scientific, Waltham, Mass.). Biofilm-forming capability of each isolate was determined by a crystal violet binding assay (Head and Yu 2004). Overnight cell cultures were diluted (1:100) in 0.1% peptone water (Becton Dickinson). Ten µl of diluted culture was dispensed into wells of 96-well polyvinyl chloride (PVC) microplates (BD Biosciences, Bedford, Mass.) containing 90 µl of LB broth and LB broth without salt (10 g tryptone and 5 g yeast extract per liter of distilled deionized water), and beef, pork, turkey, chicken, cantaloupe, lettuce, alfalfa sprout, spinach, tilapia, cod, salmon, scallop, shrimp, and oyster broths. The plates were incubated for 24 h at 25 °C. Bacterial cultures were removed, and wells of the 96-well microplates were rinsed twice with deionized water to remover loosely attached bacteria. Each well was stained with 125 µl of 0.25% crystal violet for 30 min at room temperature. The staining solution was removed, and wells were rinsed with deionized water two times. After drying, the crystal violet bound to the biofilm was solubilized with 125 µl of 70% ethanol for 30 min. The absorbance was measured at 595 nm using a microplate reader.

4.2.3 Antimicrobial susceptibility

Antibiotic resistance profiles of the isolates were examined by Kirby–Bauer disk diffusion test according to the standard procedures outlined in the Clinical and Laboratory Standards Institute guidelines (2000). Briefly, overnight cell cultures were spread evenly over Mueller-Hinton (MH) agar (Becton Dickson) with a sterile cotton swab. The tested antimicrobial disks (Becton Dickinson) included β-lactams (ampicillin, 10 μg; amoxicillin–clavulanic acid, 30 μg; cefoxitin, 30 μg; ceftiofur, 30 μg; ceftriaxone, 30 μg; and cephalothin, 30 μg), chloramphenicol (30 μg), tetracycline (30 μg), aminoglycosides (amikacin, 30 μg; gentamicin, 10 μg; kanamycin, 30 μg; and streptomycin, 10 μg), quinolone and fluoroquinolone (ciprofloxacin, 5 μg; and nalidixic acid, 30 μg), and sulfamethoxazole–trimethoprim (10 μg). Following 24 h incubation the zones of inhibition were measured and interpreted as resistant intermediate or sensitive according to manufacturer's guidelines.

4.2.4 Curli and cellulose production

Strains were screened for the ability to produce curli, a thin coiled protein structure on the surface of cells reported to bind with components in the eukaryotic extracellular matrix (Olsén and others 1993), and cellulose production as previously described (Solomon and others 2005; Uhlich and others 2006). Briefly, the production of curli was evaluated by observing the morphotype when plated on LB agar without salt (Becton Dickinson, Sparks, Md.) supplemented with 40 mg/L of Congo red (Sigma, St. Louis, Mo.) and 20 mg/L of brilliant blue (Aldrich, Milwaukee, Wis.). Isolates were streaked onto Congo red plates and incubated for 48 h at room temperature. Plates were classified as (i) red or brown, dry, and rough, indicating

positive production of curli, or (ii) smooth and white, indicating a lack of curli production. Cellulose production was determined by streaking isolates onto LB agar without salt (Becton Dickinson, Sparks, Md.) supplemented with 200 mg/L calcofluor (fluorescent brightener 28, Sigma, St. Louis, Mo.). Isolates positive for cellulose production fluoresced under UV light (302 nm).

4.2.5 Biofilm formation on food contact surfaces

The *E. coli* O157:H7 strain isolated from chicken meat (071) was overnight cultured in LB broth at 37 °C. The cultures were diluted (1:100) in 0.1% peptone water (Becton Dickinson) and inoculated into 200 ml of prepared beef, turkey, spinach, salmon, and LB broth with no salt broths at the final concentration of $10^5$ cells/ml. Aseptically, 16 ml of broths were dispensed into petri dishes (15 by 100 mm) containing stainless steel (2 by 2 in, grade 304, finish no. 4) coupon, or high density polyethylene (HDPE) (2 by 2 in, US Plastic Corp., Lima, Ohio) coupon, or Neoprene rubber (2 by 2 in). The petri dishes were incubated at 25 °C for 24 h. After incubation, the stainless steel, HDPE, or rubber chip was transferred into a sterile petri dish and rinsed twice in 16 ml of deionized water. The surfaces of stainless steel coupons, HDPE, and rubber chips were stained with 16 ml of 0.25% crystal violet for 30 min at room temperature. The staining solution was removed, and the chips were rinsed twice in 16 ml of deionized water. The crystal violet bound to the biofilm was solubilized with 5 ml of 70% ethanol for 30 min, and the absorbance was determined at 595 nm using a microplate reader (Bio-Rad).

4.2.6 Statistical analysis

One-way analysis of variance (ANOVA) was used to determine variation in biofilm formation by *E. coli* O157:H7 strains from various isolation sources. A significant difference was defined at $p<0.05$. All analyses were performed by using SPSS (SPSS 13.0.1 for Windows, SPSS Corporation, Chicago, Ill.).

## *4.3 Results and Discussion*

4.3.1 Bacterial growth and biofilm formation

Biofilm formation and bacterial growth by the fourteen *E. coli* O157:H7 isolates were observed in LB, LB without salt, meat, poultry, produce, and seafood broths. There was no variation in bacterial growth between strains (data not shown). Strains produced greater biofilm in LB without salt than LB (data not shown). Therefore, LB without salt is used to compare biofilm formation by all isolates (Figure 4-1). All tested strains of *E. coli* O157:H7 had the ability to form biofilms in LB broth without salt in some capacity. However, biofilm formations by strain 134 isolated from cattle feces, strain 071 isolated from retail chicken, and strain 072 isolated from retail beef were significantly greater than all other isolates in LB broth without salt ($p<0.05$).

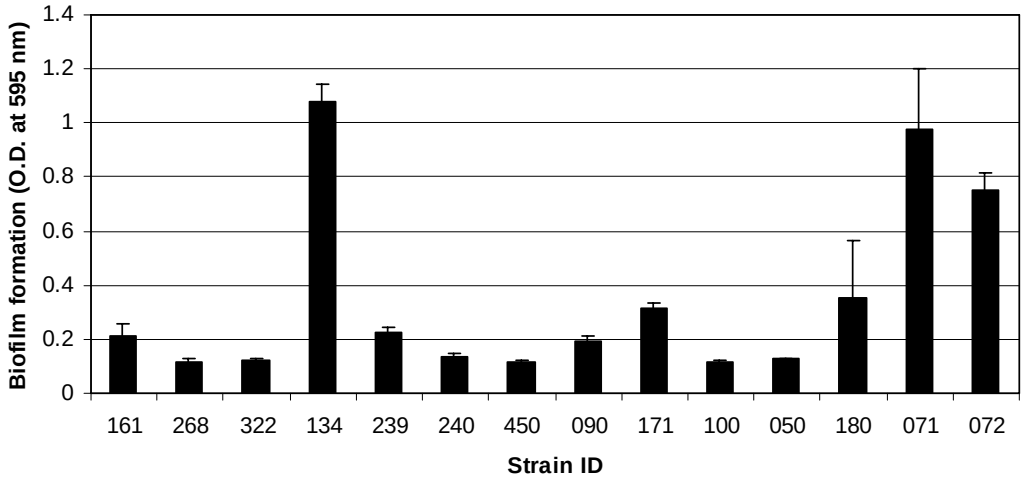



**Figure 4-1. Biofilm formation by isolates from food, animal, and human sources.** Biofilms were formed in LB broth without salt with 24 h incubation at 25 ºC. Biofilm formation was determined by crystal violet binding assay. Error bars represent the standard deviation (n=3).

Since isolates with strain ID 134, 071, and 072 produced the greatest amount of biofilm in LB without salt, biofilm formation of these three strains in raw meat, poultry, and seafood broths was compared (Figure 4-2). There was little formation of biofilm in cantaloupe, lettuce, alfalfa sprout, spinach, cod, and scallop broths by all strains (data not shown). There was no significant difference in biofilm formation by the three strains within the broths of the control LB broth without salt, beef, turkey, tilapia, and oyster ($p<0.05$). However, in pork broth, strain 071 isolated from retail chicken produced significantly greater biofilm than strains isolated from cattle feces (134) and retail beef (072; $p<0.05$). This is in direct contrast to what is observed in shrimp broths. In shrimp broth, strains 072 from retail beef and 134 isolated from cattle feces produced significantly greater biofilm than the strain 071 from retail chicken ($p<0.05$).

Formation of biofilm by strain 071 had less variation between broths than strain 134 and 072. Fish broths provided an environment for strong biofilm formation.

Strain 134 isolated from cattle feces produced significantly greater biofilm in tilapia broth than all other broths ($p<0.05$). Strain 072 isolated from retail beef produced significantly more biofilm in salmon broth than in all of the other tested broths ($p<0.05$). Strain 071 isolated from retail chicken produced the most biofilm in salmon broth when compared to other broths, however, this difference was only significant when compared to chicken and shrimp broths ($p<0.05$). This lack of variation in strain 071's ability to form biofilm in different broths emphasizes the ability of the strain isolated from retail chicken to form biofilms in many different types of broth. Biofilm formation by this strain may improve survival of other non-biofilm forming strains if a mixed biofilm were to form during processing. Biofilm forming isolates from chicken houses demonstrated the ability to enhance survival of *Campylobacter jejuni* which could not produce biofilms of their own (Trachoo and others 2002).

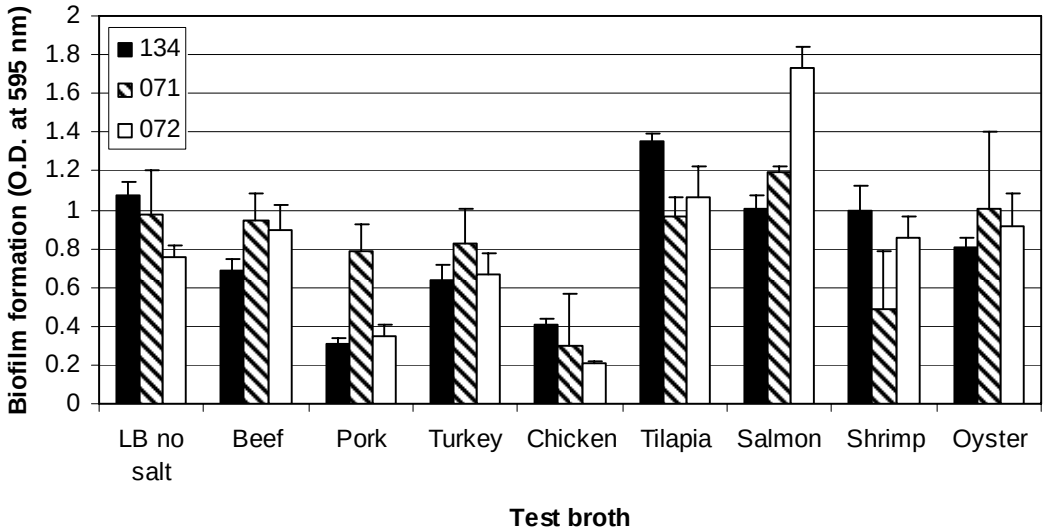



**Figure 4-2. Biofilm formation in raw meat, poultry, and seafood broths.** Strain 134 (dark bars) was isolated from cattle feces, strain 071 (hatched bars) was isolated from retail chicken, and strain 072 (light bars) was isolated from retail beef. Biofilm formation was determined by crystal violet binding assay. Error bars represent the standard deviation (n=3).

4.3.2 Antimicrobial susceptibility profiles

All isolates were tested for antimicrobial susceptibility by the Kirby-Bauer disk diffusion method. All isolated were resistant to at least one of the antimicrobials. Fourteen strains (100%) were susceptible to the β-lactam ampicillin. Eight strains (57%) were resistant to streptomycin. Eight strains (57%) were resistant to tetracycline, including strain 071 isolated from retail chicken. Strain 071 was also resistant to the quinolone nalidixic acid. Nalidixic acid resistance was only seen in strains 090, 100, and 072, all isolated from food sources, specifically turkey, pork, and chicken meat respectively.

Our finding that there is a high prevalence of ampicillin, tetracycline, and streptomycin resistance agrees with results from previous studies (Meng and others 1998; Schroeder and others 2002; Kim and Wei 2007). Lower prevalence of nalidixic acid resistance also agrees with previous studies (Zhao and others 2001).

Antimicrobial resistance can be attributed to the physical barrier created by the structure of the biofilm (Costerton and others 1999). However, we found no correlation between the number of antimicrobials a strain is resistant to and the ability of the strain to from biofilm. Therefore, there must be other mechanisms of antimicrobial resistance. The spread of antimicrobial resistance genes in *Escherichia coli* from human, animal, and food sources is primarily mediated via integrons (Zhoa and others 2001; Schroeder and others 2002). This may be particularly evident when bacteria exist in multispecies biofilms. The close physical proximity of the cells within a biofilm favors the transfer of genetic imformation (Donlan and Costerton 2002).

### 4.3.3 Curli and cellulose production

The formation of curli and cellulose were assessed on Congo red plates and calcofluor plates, respectively. Nine strains (64%) produced curli, 6 strains (43%) produced cellulose. All of the strains that produce cellulose were also curli formers. Not all strains with the ability to form curli and cellulose were able to form good biofilms, specifically strain 100 isolated from retail pork. However, the strains with the greatest ability to form biofilm (134 isolated from cattle feces, 071 from chicken meat, and 072 from retail beef) all produced both curli and cellulose. Therefore, all strains unable to produce both curli and cellulose produced significantly less biofilm in LB broth without salt ($p<0.05$). The production of curli and cellulose may not be an absolute factor in the ability of a strain to form biofilm, however our findings suggest it is one of several contributing factors.

**Table 4-1. Curli and cellulose production and antimicrobial resistance profiles of *E. coli* O157:H7 from various isolation sources.**

| Strain ID | Isolation Source | Cellulose production[a] | Curli production[b] | Resistant Antimicrobials[c] |
|---|---|---|---|---|
| 161 | Feces (human) | - | + | AMP, TET |
| 268 | Meat | - | - | AMP, STR |
| 322 | Meat | - | - | AMP, TET, STR |
| 134 | Cattle | + | + | AMP, CF, TET |
| 239 | Poultry | - | + | AMP, GEN, STR |
| 240 | Poultry | - | - | AMP, TET, STR |
| 450 | Mayonnaise | - | - | AMP, TET, STR |
| 090 | Turkey | + | + | AMP, CF, NA, KAN, TET, STR |
| 171 | Canine | - | + | AMP |
| 100 | Pork meat | + | + | AMP, CIP, NA, SXT, TET, STR |
| 050 | Chicken meat | - | - | AMP, TET, STR |
| 180 | Beef | + | + | AMP, CF |
| 071 | Chicken meat | + | + | AMP, NA, TET |
| 072 | Beef | + | + | AMP |

[a]Cellulose production was determined by fluorescence under UV light (302 nm) on LB without salt agar supplemented with calcofluor.
[b]Curli production was determined by presence of red or brown morphotypes on LB without salt agar supplemented with congo red and brilliant blue.
[c]Antimicrobial resistance profiles for Ampicillin (AMP), Tetracycline (TET), Streptomycin (STR), Cephalothin (CF), Gentamicin (GEN), Nalidixic Acid (NA), Kanamycin (KAN), Ciprofloxacin (CIP), Sulfmethoxazole/Trimethoprim (SXT) were determined by the Kirby-Bauer disk diffusion test on MH agar

4.3.4 Biofilm formation on stainless steel, rubber, and HDPE

The ability of the *E. coli* O157:H7 strain isolated from chicken meat to form biofilm was evaluated on various food contact surfaces in LB broth without salt, beef, turkey, spinach, and salmon broth (Figure 4-3). This strain was tested because of its strong ability to produce biofilm in the selected broths, ability to produce curli and cellulose, and multidrug resistance. The pathogen formed significantly greater biofilm on the surface of stainless steel and neoprene rubber in all food broth than on HDPE ($p < 0.05$). Therefore, when cleaning these surfaces measures to remove biofilm should be heavily considered. Furthermore, this exemplifies the importance of considering food safety when selecting materials for food processing equipment and surfaces.

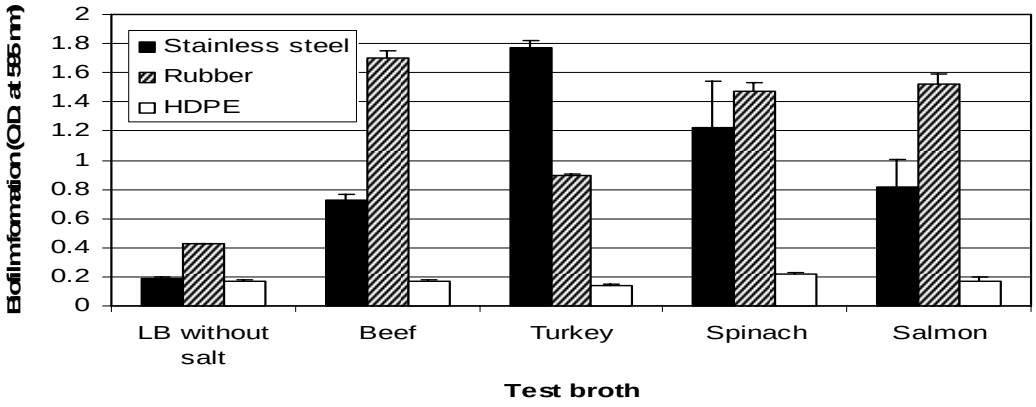



**Figure 4-3. Biofilm formation by *E. coli* O157:H7 from chicken meat on the surface of stainless steel, rubber, and HDPE in various food broths.** Biofilm was formed by the *E. coli* O157:H7 strain on stainless steel, rubber, and HDPE coupons submerged in raw beef, raw turkey, spinach, salmon, and LB without salt broths for 24 h at 25 ºC. Determination of biofilm was done by crystal violet staining. Error bars represent the standard deviation (n=3).

*4.4 Conclusions*

*E. coli* O157:H7 strains isolated from various food, animal, and human sources are able to form biofilms of different capacity. Strains from cattle feces (134),

retail chicken (071), and retail beef (072) are good biofilm-formers. All of the strong-biofilm forming strains showed resistance to ampicillin and strain 071 isolated from retail chicken also showed resistance to the quinolone nalidixic acid. Strain 071 was also able to form strong biofilm on the surface of stainless steel and neoprene rubber, two materials common in the food processing environment. The strong biofilm forming strains were able to produce curli and cellulose, indicating the production of curli and cellulose may be a contributing factor of biofilm formation by *E. coli* O157:H7.

# Chapter 5: Conclusions

Bacterial biofilms are of concern in the food processing environment. Contamination with *E. coli* O157:H7 can cost producers pricey product recalls and cost consumers serious health problems. Understanding the ability of bacteria, specifically *E. coli* O157:H7, to form biofilms in food processing facilities enables processors to take better control over prevention of contamination from *E. coli* O157:H7 biofilms.

*E. coli* O157:H7 strain from cattle was able to form significant biofilms in all tested broths at 12 and 24 hours at room temperature. The strain is able to better attach and form subsequent biofilms on stainless steel than on glass in most food material broths. Furthermore, cells attached to stainless steel are able to transfer and attach to the surface of raw meat, raw poultry, RTE deli meats, and raw produce products at concentrations able to cause intestinal disease. *E. coli* O157:H7 can persist in animal and farm, food and water, and human environments. Therefore, all sectors of food handling and processing are susceptible to biofilm as a continuous source of contamination of *E. coli* O157:H7.

*E. coli* O157:H7 isolated from food, animal, and human sources showed some capacity to form biofilm. All biofilm-forming strains showed resistance to at least one antimicrobial. Antimicrobial resistance has the potential to increase difficulty of treatment of bacterial infection. Also, mechanisms that aid in the antimicrobial

resistance of biofilms are similar to those that aid in resistance to sanitizing practices in food processing. By inhibiting biofilms formation sanitation procedures may be more effective at removing pathogens from contact surfaces. Therefore, understanding factors contributing to biofilm formation are of utmost importance. This study found little correlation between production of the quorum sensing signal AI-2 and biofilm formation. However, strains isolated from food, animal, and human sources that formed strong biofilms were also able to produce both curli and cellulose suggesting curli and cellulose may be contributing factors to biofilm formation.

*E. coli* O157:H7 is present in many sectors of the food industry and the observation that strains isolated from a variety of retail food products and animals are able to form biofilms in a variety of food broths indicates persistent contamination is not isolated on one sector of industry. Therefore, equipment design and cleaning and sanitizing procedures in food industry should always consider the prevention and removal of bacterial biofilms in order to prevent the transfer of attached bacteria as demonstrated in this study.

# List of Abbreviations

AHL- Acylhomoserine lactone
AI-2- Autoinducer-2 singals
CDC- Centers for Disease Control and Prevention
CFU- Colony forming units
CIP- Cleaning in place
DAEC- Diffusely adhering *Escherichia coli*
EAEC- Enteroaggregative *Escherichia coli*
EHEC- Enterohemorrhagic *Escherichia coli*
EIEC- Enteroinvasive *Escherichia coli*
EPEC- Enteropathogenic *Escherichia coli*
ETEC- Enterotoxigenic *Escherichia coli*
EPS- Exopolysaccharide, Extra polymeric substance
FDA- Food and Drug Administration
HDPE-High density polyethylene
HUS-Hemolytic uremic syndrome
RTE- Ready-to-eat

# Bibliography

Augustin M, Ali-Vehmas T. 2004. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. J Pharm Pharmaceut Sci 7:55-64.

Barkocy-Gallagher GA, Arthur TM, Siragusa GR, Keen JE, Elder RO, Laegreid WW, Koohmaraie M. 2001. Genotypic analyses of *Escherichia coli* O157:H7 and O157 nonmotile isolates recovered from beef cattle and carcasses at processing plants in the Midwestern states of the Unites States. Appl Environ Microbiol 67:3810-3818.

Barnhart MM, Chapman MR. 2006. Curli biogenesis and function. Annu Rev Microbiol 60:131-47.

Besser RE, Griffin PM, Slutsker L. 1999. *Escherichia coli* O157:H7 gastroenteritis and the hemolytic uremic syndrome: an emerging infectious disease. Annu Rev Med 50:355-367.

Boyce TG, Swerdlow DL, Griffin PM. 1995. Current concepts: *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. N Engl J Med 333:364-368.

Boyer RR, Sumner SS, Williams RC, Pierson MD, Popham DL, Kniel KE. 2007. Influence of curli expression by *Escherichia coli* O157:H7 on the cell's overall hydrophobicity, charge, and ability to attach to lettuce. J Food Prot 70:1339-1345.

Buzby JC, T Roberts, C-TJ Lin, JM MacDonald. 1996. Bacterial foodborne disease: medical costs and productivity losses. Economic Research Service. Agricultural Economic Report No. 741. U.S. Department of Agriculture, Washington, D.C.

Buzby JC, T Roberts. 1998. Economic costs and trade impacts of microbial foodborne illness. World Health Stat Quart 50:57-66.

Campbell GR, Prosser L, Glover A, Killham K. 2001. Detection of *E. coli* O157:H7 in soil and water using multiplex PCR. J Appl Microbiol 91:1004-1010.

Centers for Disease Control and Prevention. 1993. Update: Multistate outbreak of *Escherichia coli* O157:H7 infections from hamburgers-Western United States, 1992-1993. MMWR 42:258-263.

Centers for Disease Control and Prevention. 2006a. Update on Multi-State Outbreak of *E. coli* O157:H7 Infections from Fresh Spinach, October 6, 2006 [Internet: cited 2006 December 21]. Available from: http://www.cdc.gov/ecoli/2006/september/updates/100606.htm.

Centers for Disease Control and Prevention. 2006b. Multistate Outbreak of *E. coli* O157:H7 Infections, November-December 2006 [Internet: cited 2006 December 21]. Available from: http://www.cdc.gov/ecoli/2006/december/121406.htm.

Charmichael I, Harper IS, Coventry MJ, Taylor PWJ, Wan J, Hickey MW. 1999. Bacterial colonization and biofilm formation on minimally processed vegetables. J Appl Micrbiol Symp Suppl 85(2):45S-51S.

Chmielewski RAN, Frank JF. 2003. Biofilm formation and control in food processing facilities. Comp Rev Food Sci Food Safety 2: 22-32.

Clinical and Laboratory Standards Institute/NCCLS. 2000. Performance standards for antimicrobial disk susceptibility tests, 7th ed. Approved standard M2-A7. National Committee for Clinical Laboratory Standards, Wayne, Pa.

Costerton JW, Stewert PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318-1322.

Dean-Nystrom EA, Melton-Celsa AR, Pohlenz JFL, Moon HW, and O'Brien AD. 2003. Comparative pathogenicity of *Escherichia coli* O157 and intimin-negative non-O157 shiga toxin-producing *E. coli* strains in neonatal pigs. Infect Immun 71:6526-6533.

Dewanti R, Wong ACL. 1995. Influence of culture conditions on biofilm formation by *Escherichia coli* O157:H7. Int J Food Microbiol 26:147-164.

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

Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167-193.

Fatemi P, Frank JF. 1999. Inactivation of *Listeria monocytogenes/Pseudomonas* biofilms by peracid sanitizers. J Food Prot 62:761-765.

Feng P, Weagant SD. Bacteriological Analytical Manual Online. Diarrheagenic *Escherichia coli*. FDA: Center for Food Safety and Applied Nutrition [Internet]. 2002 [cited 2007 August 15]. Available from: http://www.cfsan.fda.gov/~ebam/bam-4a.html.

Food and Drug Administration. 1992. Aerobic plate count, p.17-26. In FDA bacteriological analytical manual, 7th ed. AOAC International, Arlington, Va.

Fratamico PM, Bagi LK, Pepe T. 2000. A multiplex polymerase chain reaction assay for rapid detection and identification of *Escherichia coli* O157:H7 in foods and bovine feces. J Food Prot 63:1032-1037.

González Barrios AF, Zuo R, Hashimoto Y, Yang L, Bentley WE, Wood TK. 2006. Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). J Bacteriol 188:305-316.

Hall-Stoodley L, Costerton JW, Stoodley P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nature Rev Microbiol 2:95-108.

Head NE, Yu H. 2004, Cross-sectional analysis of clinical and environmental isolates of *Pseudomonas aeruginosa*: biofilm-formation, virulence, and genome diversity. Infect Immun 72:133-144.

Kim S-H, Wei C-I. 2007. Biofilm formation by multidrug-resistant *Salmonella enterica* serotype Typhimurium phage type DT104 and other pathogens. J Food Prot 70:22-29.

Kumar CG, Anand SK. 1998. Significance of microbial biofilms in food industry: a review. Int J Food Microbiol 42:9-27.

LeCercq-Perat MN, Lalande M. 1994. Cleanability in relation to surface chemical composition and surface finishing of some materials commonly used in food industries. J Food Eng 23:501-517.

McGee P, Bolton DJ, Sheridan JJ, Earley B, Kelly G, Leonard N. 2002. Survival of Escherichia coli O157:H7 in farm water: its role as a vector in the transmission of the organism within herds. J Appl Microbiol 93:706-713.

Mead PS, Griffin PM. 1998. *Escherichia coli* O157:H7. Lancet 352:1207-1212.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. 1999. Food-related illness and death in the United States. Emerg Infect Dis 5:607-625.

Meng J, Zhao S, Doyle MP. 1998a. Virulence genes of Shiga toxin-producing *Escherichia coli* isolated from food, animals, and humans. Int J Food Microbiol 45:229-235.

Meng J, Zhao S, Doyle MP, Joseph SW. 1998b. Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food, and humans. J Food Prot 61:1511-1514.

Midelet G, Carpentier B. 2002. Transfer of microorganisms, including *Listeria monocytogenes*, from various materials to beef. Appl Environ Microbiol 68:4015-4024.

Montville TJ, Matthews KR. 2005. Food Microbiology: an Introduction. Washington (DC): ASM Press. 380 p.

Morris CE, Monier JM, Jacques MA. 1997. Methods for observing microbial biofilms directly on leaf surfaces and recovering them for isolation of culturable microorganisms. Appl Environ Microbiol 63:1570-1576.

Moxley RA. 2004. *Escherichia coli* O157:H7: and update on intestinal colonization and virulence mechanisms. Anim Health Res Rev 5(1):15-33.

Murinda SE, Nguyen LT, Landers TL, Draughon FA, Mathew AG, Hogan JS, Smith KL, Hancock DD, Oliver SP. 2004. Comparison on *Escherichia coli* isolates from humans, food, and farm and companion animals for presence of shiga toxin-producing *E. coli* virulence markers. Foodborne Pathog Dis 1:178-184.

Mustapha A, Liewen MB. 1989. Destruction of *Listeria monocytogenes* by sodium hypochlorite and quaternary ammonium sanitizers. J Food Prot 52:306-311.

Olsén A, Arnqvist A, Hammar M, Normark S. 1993. Environmental regulation of curli production in *Escherichia coli*. Infect Agents Dis 2:272-274.

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

Pillai SD, Jesudhasan PR. 2006. Quorum Sensing: How Bacteria Communicate. Food Technol 60(4):42-50.

Phillips B, Tyerman K, Whiteley SM. 2005. Use of antibiotics in suspected haemolytic uraemic syndrome. BMJ 330:409-410.

Rangel JM, Sparling PH, Crowe C, Griffin PM, Swedlow DL. 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. Emerge Infect Diseases 11:603-609.

Rivas L, Fegan N, Dykes GA. 2007. Attachment of Shiga toxin *Escherichia coli* to stainless steel. Int J Food Microbiol 115:89-94.

Rodríguez A, McLandsborough LA. 2007. Evaluation of the transfer of *Listeria monocytogenes* from stainless steel and high-density polyethylene to bologna and American cheese. J Food Prot 70:600-606.

Salin V, NH Hooker. 2001. Stock market reaction to food recalls. Rev Agr Econ 23:33-46.

Schroeder CM, Zhao C, DebRoy C, Torcolini J, Zhao S, White DG, Wagner DD, McDermott PF, Walker RD, Meng J. 2002. Antimicrobial resistance of *Escherichia coli* O157:H7 isolated from humans, cattle, swine, and food. Appl Environ Microbiol 68:576-581.

Sivapalasingam S, Friedman CR, Cohen L, Tauxe RV. 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J Food Prot 67:2342-2353.

Solano C, García B, Valle J, Berasain C, Ghigo J-M, Gamazo C, Lasa I. 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. Mol Microbiol 43:793-808.

Solomon EB, Niemira BA, Sapers GM, Annous BA. 2005. Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. J Food Prot 68:906-912.

Surette MG, Bassler BL. 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proc Natl Acad Sci USA 95:7046-7050.

Tarr PI, Gordon CA, Chandler WL. 2005. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet 365:1073-1086.

Thakur S. 2007. *Escherichia coli*. College Park (MD): University of Maryland Department of Nutrition and Food Science. 12 p.

Trachoo N, Frank JF, Stern NJ. 2002. Survival of *Campylobacter jejuni* in biofilms isolated from chicken houses. J Food Prot 65:1110-1116.

Uhlich GA, Cooke PH, Solomon EB. 2006. Analysis of the red-dry-rough phenotype of an *Escherichia coli* O157:H7 strain and its role in biofilm formation and resistance to antibacterial agents. Appl Environ Microbiol 72:2564-2572.

Van Houdt R, Aertsen A, Jansen A, Quintana AL, Michiels CW. 2004. Biofilm formatioin and cell-to-cell signaling in Gram-negative bacteria isolated from a food processing environment. J Appl Microbiol 96:177-184.

Wall Street Journal Staff. 2007. Topps Meat Co. closes a week after huge recall. Wall Street Journal, October 6, Eastern Edition. http://www.proquest.com/ (accessed October 28, 2007)

Zhao S, White DG, Ge B, Ayers S, Friedman S, English L, Wagner D, Gaines S, Meng J. 2001. Identification and characterization of integron-mediated antibiotic

resistance among Shiga toxin-producing *Escherichia coli* isolates. Appl Environ Microbiol 67:1558-1564.

Zogaj X, Nimtz M, Rohde M, Bokranz W, Römling. 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol Microbiol 39:1452-1463.