

**DETECTION AND SURVIVAL OF *ESCHERICHIA COLI* O157:H7 IN CATTLE  
WATER TROUGHS AND THE EFFECTS OF CETYLPYRIDINIUM CHLORIDE  
AGAINST *ESCHERICHIA COLI* O157:H7 BIOFILMS ON THE SURFACE OF  
STAINLESS STEEL**

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## ABSTRACT

*Escherichia coli* O157:H7 is an emerging food pathogen that was first identified as a cause of illness in 1982. According to CDC (The Center for Disease Control and Prevention) estimates, *E. coli* O157:H7 is responsible for about 73,000 illnesses, 2,000 hospitalizations and 60 deaths in the United States each year. Cattle are the principal reservoir of *E. coli* O157:H7. Contamination of feed and drinking water with cattle feces is an importance factor in the prevalence of infection in cattle which, in turn, results in contamination of food and the environment. *Escherichia coli* O157:H7 is a very persistent pathogen and has been shown to survive for long periods of time in the cattle farm environments. The purpose of this study was to test for the presence of *E. coli* O157:H7 in cattle water troughs of four cattle farms in Louisiana and also to study the survival and growth characteristics of different strains of *E. coli* O157:H7 in cattle water trough sediments using experimental microcosms. *Escherichia coli* O157:H7 can form biofilms on food contact surfaces and is responsible for several outbreaks caused due to cross-contamination in food processing plants. An additional objective of this study was to investigate the antimicrobial effects of cetylpyridinium chloride (CPC) against *E. coli* O157:H7 biofilms grown on stainless steel surfaces in different temperature and culture conditions. Results from this study showed an *E. coli* O157:H7 prevalence of 4.5% in the cattle water troughs. Survival studies showed variability in the growth of *E. coli* O157:H7 at different temperatures. Higher temperatures (25°C and 37°C) resulted in greater decrease of *E. coli* O157:H7 than at lower temperatures (5°C and 15°C), especially in the presence of natural microflora. It was also observed that the growth and survival of human *E. coli* O157:H7 isolates was significantly lower than environmental isolates at

lower temperatures (5°C and 15°C). The biofilm study showed that <1.0% CPC was effective in inactivation of *E. coli* O157:H7 biofilms grown on stainless steel in all treatment conditions.

**CHAPTER 1**  
**GENERAL INTRODUCTION**

*Escherichia coli* O157:H7 is an emerging food pathogen with a worldwide distribution. It has emerged as an important pathogen since it was first identified as a cause of illness in 1982 during an outbreak of severe bloody diarrhea due to contaminated hamburgers (Riley et al., 1983). The frequency of illnesses associated with *E. coli* O157:H7 including hemorrhagic colitis, hemolytic uremic syndrome, thrombocytopenic purpura has been increasing during the past two decades. The CDC (The Center for Disease Control and Prevention) estimates that *E. coli* O157:H7 is responsible for about 73,000 illnesses, 2,000 hospitalizations and 60 deaths every year (Rangel et al., 2005). Also, according to CDC estimates, the annual cost of illness due to *E. coli* O157:H7 infections acquired from food or other sources is \$405 million (Frenzen et al., 2005).

Foods that have most commonly been implicated in *E. coli* O157:H7 outbreaks are hamburger meat, apples, unpasteurized apple juice, milk, potatoes, lettuce, spinach, water and mayonnaise (Deisingh and Thompson, 2004). Cattle are believed to be the principal reservoir of *E. coli* O157:H7 and shed this organism in their feces. Studies have shown that contamination of feed and drinking water with cattle feces may be an important factor in the prevalence of infection in cattle and which results in contamination of the environment and of beef products during processing (LeJeune, 2001).

Although several factors, such as competition, predation and low nutrient concentrations could considerably reduce the survival rates of bacteria in the aquatic environment, *E. coli* have been shown to survive and grow in sediments (Pommepuy et al., 1992; Davies et al., 1995). Studies indicate that *E. coli* O157:H7 is very persistent and can be isolated for long periods of time from the cattle farm environments (Hancock et

al., 1997). Therefore, it is possible that water trough sediments contaminated with *E. coli* O157:H7 from cattle feces may persist and serve as long-term reservoirs of the pathogen on farms and act as a continuous source of infection for cattle. It is believed that improving the sanitation on the farm to reduce fecal contamination of water troughs, cattle feed, and fecal soiling of hides could reduce environmental contamination and contamination of beef products at slaughter (LeJeune et al., 2001). Most research on *E. coli* O157:H7 has concentrated on understanding the ecology of *E. coli* O157:H7 in cattle; however there is very limited research on the ecology of *E. coli* O157:H7 in cattle farm environment that might help in developing hazard analysis and critical control point (HACCP) programs for producers to reduce these pathogen on farms.

Microbial biofilm growth is a widespread phenomenon in the food industry (Boulangue-Petermann, 1996). Biofilm formation on food contact surfaces in processing plants are known to result in increased resistance of cells to disinfectants and can be a source of cross-contamination with foodborne pathogens such as *E. coli* O157:H7 (Notermans et al., 1991; Kumar and Anand, 1998). Beef and beef products have been most frequently implicated in outbreaks of foodborne *E. coli* O157 infection due to cross-contamination from animal feces, hide, or the intestine during processing. *Escherichia coli* O157:H7 is known to produce extracellular polymeric substances (EPS) and form biofilms on surfaces (Junkins and Doyle, 1992). Quaternary ammonium compounds (QACs) are frequently used as disinfectants in food industries to prevent the spread of microorganisms (Sharma and Beuchat, 2004; Breen et al., 1995). Cetylpyridinium chloride (CPC) is a quaternary ammonium compound that has been used for over 50 years in oral hygiene products and has shown to be successful in reducing biofilms on

some food surfaces (Breen et al., 1997). However, there has been no published research on the effects of CPC on *E. coli* O157:H7 biofilms on food contact surfaces such as stainless steel.

The objectives of this study were to: (1) investigate the prevalence and characterize *E. coli* O157:H7 from cattle water troughs in Louisiana, (2) investigate the survival and growth characteristics of different strains of *E. coli* O157:H7 at different temperatures in microcosms simulating cattle water troughs, (3) determine the effects of temperature and culture conditions on biofilm formation by *E. coli* O157:H7 on stainless steel surfaces and to determine the effects of cetylpyridinium chloride on these biofilms.

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**CHAPTER 2**  
**LITERATURE REVIEW**

## ***ESCHERICHIA COLI***

*Escherichia coli* was first identified in 1885 by the German pediatrician Theodor Escherich from children's feces. It is a normal and essential part of the flora of the intestine tract of humans and warm-blooded animals and helps maintain the physiology of the healthy host. *Escherichia coli* is a member of the family Enterobacteriaceae, which include facultatively anaerobic Gram-negative, usually catalase-positive, usually nitrate-reducing, and motile by peritrichous flagella or nonmotile bacteria.

*E. coli* is divided into serogroups and serotypes based on differences in the antigens on the surface of the bacterial cell, O-outer membrane antigens, H- flagella antigens and k capsule antigens. There are more than 170 different serogroups of *E. coli* based on somatic O antigens and over 50 flagella antigens and 100 capsular antigens are recognized and used to further subdivide *E. coli* into serotypes. Serogrouping and serotyping along with other information on other virulence factors such as phage type and toxin production is now used to distinguish strains that can cause infectious diseases in humans and animals.

*E. coli* is often used as an indicator of fecal contamination because it is abundant in human and animal feces and not usually found in other niches. It is used to indicate unsanitary processing in the food-processing environment (Bell and Kyriakides, 1998). Most strains of *E. coli* are not pathogens; however, they can be opportunistic pathogens that cause infections such as Gram-negative sepsis, urinary tract infection, pneumonia in immunocompromised people, and meningitis (Deisingh and Thompson, 2004). There are also pathogenic groups of *E. coli* called diarrheagenic *E. coli* that when ingested cause gastrointestinal illness in healthy humans. These groups of *E. coli* are classified based on

their unique virulence factors and include the following pathogenic groups:

enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC). Of these, only the first 4 groups have been implicated in food or water borne illness (Tarr, 1995).

The term enterohemorrhagic *E. coli* refers to the serotypes of *E. coli* that cause hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS), produce one or more phage-encoded Shiga toxins (Stx), possess a 60-megadalton virulence plasmid, and produce attaching and effacing lesions. The term shiga toxin producing *E. coli* (STEC) or verotoxigenic *E. coli* (VTEC) is used to refer to strains of *E. coli* that produce Shiga toxins without referring to any clinical symptoms produced by them. Although >100 different serotypes of *E. coli* have been reported to produce Stx, only those that have been clinically associated with HC are designated as EHEC. Of these, *E. coli* O157:H7 is now recognized as an important agent of foodborne human diseases with worldwide distribution (Armstrong et al., 1996; Tarr, 1995; Griffin and Tauxe, 1991; Neill et al., 1987). Thus enterohemorrhagic *E. coli* are a subset of Shiga toxin producing *E. coli*.

### **VIRULENCE FACTORS OF *E. COLI* O157:H7**

*Escherichia coli* O157:H7 is the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome (HUS) in the U.S. Toxins produced by EHEC strains are called verotoxins, Shiga-like toxins or Shiga toxins (Stx). These toxins are believed to be the major factors contributing to the pathogenesis of HUS, although the O157 lipopolysaccharide may also contribute to this disease syndrome (Karpman et al., 1997). *Escherichia coli* O157:H7 can produce 2 major immunologically distinct toxins, Stx1, Stx2, or both. Shiga toxins

resemble the Shiga toxin produced by *Shigella dysenteriae* type 1 in both structure and activity. Shiga toxin of *Shigella dysenteriae* is nearly identical in structure and function to Stx1 of *E. coli* O157:H7, differing by only one amino acid. Whereas, Stx2 exhibits 58% nucleotide homology and 56% amino acid homology to Stx1 (Jackson et al., 1987).

Shiga toxin is an A-B toxin in which the A subunit contains the enzymatically active molecule while the B subunit binds the holotoxin to the target eukaryotic cell. The B subunits of both Stx1 and Stx2 bind to a specific glycolipid on eukaryotic cell called the globotriaosylceramide, or gal( $\alpha$ )1-4gal( $\beta$ )1-4 glucosylceramide. This surface receptor is considered to be the chief determinant of cellular sensitivity and the organ distribution of which is believed to be responsible for extraintestinal organ injury secondary to the gastrointestinal symptoms of *E. coli* O157:H7 infection. These toxins are believed to induce endothelial damage in the small blood vessels supplying the kidney, gastrointestinal tract, central nervous system and various other organs, thus giving rise to the signs and symptoms that characterize HUS (Arbus 1997). This receptor is also present on Vero cells and other cell lines sensitive to Shiga-like toxin (Boyd and Lingwood, 1989). After internalization, the A subunit is cleaved to A<sub>1</sub> and A<sub>2</sub> fragments. The A<sub>1</sub> fragment binds to a specific adenine (A4324) on 28S rRNA on the 60S ribosomal subunit, resulting in disruption of ribosomal structural integrity and protein synthesis (O'Brien et al., 1992). *E. coli* O157:H7 is not pathogenic in cattle because they lack the globotriaosylceramide receptor in their gastrointestinal tract (Pruimboom-Brees, et al., 2000).

The *stx* genes are encoded by  $\lambda$ -like phages suggesting that *E. coli* O157:H7 may have acquired these toxins through phage-mediated genetic transfer (Strockbine et al.,

1986). These two *stx* genes are located in prophages integrated into the bacterial genome with toxin production under the control of the bacteriophage late genes. As a result, when the prophage is in its latent state, toxin production is shut off, but when the prophage is induced, toxin production increases. These dormant bacteriophages can be activated from their latent state by DNA damaging agents (such as quinolones, mitomycin C, norfloxacin) or in response to signals present in the gastrointestinal tract (Acheson et al., 1998). The released virus particles can then infect susceptible *E. coli* hosts, creating new strains of toxin-producing cells. The DNA-damaging agents also can induce expression and release of toxin from its dormant state causing serious consequences to the host (Zhang et al., 2000). Therefore, antimicrobials that cause DNA damage in the bacterial cell (i.e. quinolones) cannot be used for treatment of *E. coli* O157:H7 infection.

*Escherichia coli* O157:H7 can also produce virulence factors other than Shiga toxin such as the locus for enterocyte effacement (LEE) and a large molecular weight plasmid that encodes for a hemolysin. The LEE is a large cluster of genes that are responsible for the intimate attachment of the bacterium to the apical membrane of the enterocyte and subsequent destruction or effacement of the microvilli. The intimate attachment of the bacterial cell to the epithelium is attributed to the adhesion protein, intimin, which mediates intimate attachment to epithelial cells by actin aggregation, and Tir, a bacterial protein, which is inserted into the host membrane and serves as the receptor for intimin. Intimin is an outer membrane protein encoded by the chromosomal *eae* gene (Tarr, 1995).

*Escherichia coli* O157:H7 also possesses a large molecular weight plasmid (~90 kilobase) that contains several virulence genes, including a pore-forming hemolysin also

known as enterohemolysin or EHEC hemolysin. The direct contribution of this substance to virulence is not known (Schmidt et al., 1995).

### **EPIDEMIOLOGY OF *E. COLI* O157:H7**

Foodborne illness is a major public health problem in the United States and worldwide. According to recent estimates 76 million illnesses and 5,000 deaths are attributed annually to foodborne illness in the US (CDC). It is estimated that 73,000 illnesses occur each year due to *E. coli* O157:H7 infection in the United States, leading to an estimated 2,000 hospitalizations and 60 deaths annually (Rangel et al., 2005).

*Escherichia coli* O157:H7 was first associated with hemorrhagic colitis in humans in 1982 (Riley et al., 1983) and was later isolated from cattle (Chapman et al., 1989; Chapman et al., 1993; Wells et al., 1991). *Escherichia coli* O157:H7 was recognized as an important pathogen of concern after the 1993 large multistate *E. coli* O157:H7 outbreak linked to undercooked ground beef patties sold from a fast-food restaurant chain in which more than 700 persons became ill (Bell et al., 1994; Tuttle et al., 1999).

Illnesses associated with *E. coli* O157:H7 including hemorrhagic colitis, hemolytic uremic syndrome, thrombocytopenic purpura have been reported with increasing frequency during the past two decades. Hemorrhagic colitis caused by *E. coli* O157:H7 is characterized by severe abdominal cramps, bloody stools, little or no fever and evidence of colonic mucosal edema. About 5% of infected persons develop hemolytic uremic syndrome characterized by hemolytic anemia, thrombocytopenia, renal failure and a death rate of 3-5%, with its most severe manifestations in young children and the elderly (Tarr, 1995). Severe gastrointestinal disease followed by HC may also

have adverse effects on the central nervous system, pancreas, lungs and heart (Deisingh and Thompson, 2004).

The infectious dose for *E. coli* O157:H7 is estimated to be small, ranging from 10 to 100 cells (Bell et al., 1994). *Escherichia coli* O157:H7 infections are mostly food or water borne and the foods that have been implicated are undercooked ground beef (MacDonald et al., 1988; Bryant et al., 1989; Doyle and Schoeni et al., 1987; Willshaw et al., 1993), milk and milk products (Wells et al., 1991; Morgan et al., 1993), drinking water (Swerdlow et al., 1992), unpasteurized apple juice (Conner and Kotrola, 1995), cold sandwiches, sprouts and vegetables (Cater et al., 1987; Morgan et al., 1988; Besser et al., 1993; Abdul-Raouf et al., 1993; Armstrong et al., 1996). The most recent *E. coli* O157:H7 outbreak in August-September 2006 which resulted in 199 sick people and 2 deaths occurred due to contaminated spinach (FDA, 2006). However, most outbreaks of *E. coli* O157:H7 have been the result of transmission through foods of bovine origin. In cases involving non-bovine foods, cross contamination by beef or contamination with bovine feces has often been suspected. *Escherichia coli* O157:H7 from the intestinal tract of healthy cattle contaminates meat during slaughter and processing (Wells et al., 1991; Wang et al., 1996).

Contaminated water in lakes, ponds, and swimming pools has also been a major source of *E. coli* O157:H7 outbreaks (Chalmers et al., 2000). Several outbreaks have been associated with a visit to a country fair, farm, or petting zoo following contact with carrier animals, especially in children. Person-to-person transmission has also been reported as a source of infection in child-care centers (Sanchez et al., 2002). Since there

is lack of treatment to decrease the severity of illness or to prevent complications, prevention of *E. coli* O157:H7 infections is very critical.

### **ECOLOGY OF *E. COLI* O157:H7**

As there is considerable evidence to show that cattle are the main reservoir of *E. coli* O157:H7, many studies have focused on the epidemiology of this organism in cattle farms (Hancock et al., 1997; Hancock et al., 1994; Chapman et al., 1993; Garber et al., 1995). *Escherichia coli* O157:H7 is a transient member of the normal flora of cattle with a prevalence rate of <1 to 5% to as high as 13 to 28% of animals in many studies worldwide (Sanchez et al., 2002). The range of duration of *E. coli* O157:H7 in animals can vary from a few days to a year (Zhao et al., 1995) depending on many factors such as diet, drinking water contamination, competing microbial flora, immune response, age, breed, *E. coli* strain, housing conditions, and season. Warm summer months have also been associated with increasing rates of *E. coli* O157:H7 fecal shedding. Other animals on the farm such as goats, sheep, and swine have also been found to be carriers of *E. coli* O157:H7 (Sanchez et al., 2002).

*E. coli* O157:H7 can be introduced into the feedlot by various sources such as cattle feed, contaminated water, and other animals (wild or domestic). *Escherichia coli* O157:H7 is not pathogenic in cattle and is shed in the feces of healthy cattle (Sanchez et al., 2002). Bovine feces containing *E. coli* O157:H7 could be a source of environmental contamination. Studies on the fate of *E. coli* O157:H7 in bovine feces revealed that this pathogen can survive for several weeks in the farm environment, depending on temperature and water activity (Hancock et al., 1994; Chapman et al., 1993). Cattle shed *E. coli* O157:H7 in their feces only transiently for periods lasting 1 to 3 months or less;

however *E. coli* O157:H7 can persist on individual farms for up to 2 years (Mechie et al., 1997). Feed has also been reported as a vehicle for transmission of *E. coli* O157:H7 in cattle (Davis et al., 2003). Feces and water contaminated with *E. coli* O157:H7 can contaminate pastures and crops where it can survive and serve as a source of contamination of feed (Maule, 2000). It is possible that *E. coli* O157:H7 can survive for months in environmental sources such as feed and water and may play a role in transmission within and between farms and thus act as a source of infection of humans even in the absence of direct contact with animals (Hancock et al., 1997). It is, therefore, believed that manure management, feed bunk and water trough sanitation, and feed management can prevent transmission of *E. coli* O157:H7 in the cattle farm environment (Sanchez et al., 2002).

Waterborne transmission of *E. coli* O157:H7 is an emerging concern to human health as well as a source of infection of cattle (Chalmers et al., 2000; Sanchez et al., 2002). Various studies have shown that the survival times of *E. coli* in water can vary greatly, depending on the type of water (temperature, tropical, fresh, estuarine, and well water), variations in the chemical composition or carbon content of waters, and storage temperature. According to one study, it has been shown that *E. coli* O157:H7 can survive for a long period of time (12 weeks), in water especially at cold temperatures (Wang and Doyle, 1998).

Sediments have been found to provide an environment in which *E. coli* have sufficient nutrients to survive and multiply (Davies et al., 1995). Accumulation of indicator bacteria and viruses in sediments is well documented and has been attributed to the sorption of the microorganism to particles suspended in water, which then sediment

out. Sediments may contain 100 to 1000 times as many fecal indicator bacteria as the overlying water (Pommepuy et al., 1992).

Contaminated water troughs with sediments provide an environment for survival, proliferation, and horizontal spread of *E. coli* O157:H7 (Hancock et al., 1997). In natural conditions, other physical and chemical factors such as sunlight and organic matter also affect bacterial survival (Davies and Evison, 1991; Korhonen and Martikainen, 1991; Lim and Flint, 1989). Solar radiation does not reduce the total number of enteric bacteria present in aquatic ecosystems; however, increases in temperature have been related to greater decreases of culturable enteric bacteria in aquatic ecosystems (Davenport et al.; 1976; Barcina et al., 1986). The high prevalence of *E. coli* O157:H7 in water troughs suggests that the water troughs may serve as reservoirs for this the organism (LeJeune et al., 2001a). Although *E. coli* are considered to be obligate enteric parasites which can survive only transiently in the environment, various strains of *E. coli*, including *E. coli* O157:H7, have been shown to multiply efficiently in the environment in presence of moisture and nutrients (Lynn et al., 1998). Marine sediments also have been found to provide sufficient nutrients for *E. coli* to survive and multiply (Davies et al., 1995).

#### **DETECTION METHODS FOR *E. COLI* O157:H7**

*Escherichia coli* O157:H7 are phenotypically distinct from other *E. coli* in that they exhibit slow or no fermentation of sorbitol and do not have glucuronidase activity. These traits are often used to isolate this pathogen from foods. Identification of *E. coli* O157:H7 involves growth in an enrichment medium which increases low concentration of cells to detectable levels before plating on sorbitol-MacConkey agar (SMAC). The presumptive *E. coli* O157:H7 colonies are colorless on SMAC due to delayed

fermentation of sorbitol while the other members of Enterobacteriaceae appear as pink colonies (Tortorello et al., 1996; March and Ratnam, 1986). Enrichment broths containing peptone, vancomycin, cefixime, cefsulodin, and potassium tellurite are also used to enhance the detection by providing nutrients that allow *E. coli* O157:H7 to grow while inhibiting the growth of other bacteria (Deisingh and Thompson, 2004). The colorless colonies on SMAC are then confirmed by serological and biochemical methods. Several biochemical methods are used to differentiate *E. coli* from other members of Enterobacteriaceae. These include the IMViC test: the ability to produce indole from tryptophan (I), produce enough acid to reduce the medium pH below 4.4, the break point of methyl red indicator (M), produce acetoin (V) and the ability to utilize citrate (C). Latex agglutination tests are used to detect the presence of either antibody or antigen in a sample (Deisingh and Thompson, 2004).

However, isolation of *E. coli* O157:H7 from water and other environmental samples is laborious. Culturing is difficult due to large numbers of other flora that either overgrow or look like the non-sorbitol-fermenting *E. coli* O157:H7. Recently, immunomagnetic separation (IMS) that uses magnetic beads coated with antibody against *E. coli* O157:H7 has helped in improving recovery by providing an antibody-based concentration procedure from a range of matrices (Lejeune et al., 2001b). In immunomagnetic separation, polystyrene-based particles ranging from 2.8 to 4.5  $\mu\text{m}$  are used. These particles contain  $\text{Fe}_2\text{O}_4$  and  $\text{Fe}_3\text{O}_4$  to make them superparamagnetic that exhibit magnetic properties only in the presence of a magnetic separator. In this technique, immunomagnetic particles specific for the target organism are suspended and mixed with the sample. After incubation, the particles with bound *E. coli* O157:H7 cells

are separated from the suspension with a magnetic particle separator. The magnetic articles are then washed several times and plated on selective media (Safarik et al. 1995).

Several molecular techniques have been used for genotyping or subtyping *E. coli* O157:H7 and other pathogens to investigate the sources of the organisms in disease outbreaks. These techniques, including Shiga toxin genotyping, plasmid profile analysis, restriction enzyme digestion and electrophoresis of plasmid and whole cell DNA, multilocus enzyme electrophoretic typing, and phage typing, pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD) have also been used in investigations of *E. coli* O157:H7 in cattle production environments for epidemiological typing, and determining genetic relatedness between different strains (Faith et al., 1996; Rice et al., 1999; Shere et al., 1998). Random amplification of polymorphic DNA (RAPD) is a modification of the polymerase chain reaction (PCR) in which a single primer able to anneal and prime at multiple locations throughout the genome can produce a genetic fingerprint characteristic of the template DNA and of great epidemiological value (Welsh and McClelland 1990).

#### **MICROBIAL BIOFILMS: MECHANISMS OF BIOFILM FORMATION**

A biofilm is an association of microorganisms attached to a surface and embedded in a matrix of the extracellular polymeric substances (EPS) produced by the microorganisms (Costerton et al., 1995). The role of biofilm formation has been very well studied in different habitats (Zottola and Sasahara, 1994). In natural and industrial environments, bacteria are commonly found not as free-living cells but attached to surfaces in the form of biofilms (Costerton et al., 1995).

Biofilm formation is a dynamic and a multistep process. The transition from planktonic growth to adhesion to a surface is triggered by different environmental signals in different organisms. For instance, *P. aeruginosa* forms biofilms under most conditions that allow growth, and some strains of *E. coli* K-12 cannot form biofilms in minimal medium unless supplemented with amino acids (Costerton et al., 1995), whereas *E. coli* O157:H7 has been reported to make a biofilm only under low-nutrient conditions (Dewanti and Wong, 1995). Biofilm formation includes the following four steps: conditioning, adhesion, microcolony formation, and biofilm maturation (Marshall 1992). In the first stage, bacteria present in food processing environments are adsorbed to the surface along with other organic and inorganic molecules to form a conditioned film. The conditioning alters the physico-chemical properties of the surface, such as, surface free energy, changes in hydrophobicity and electrostatic charges which may also affect the subsequent sequence of microbial events (Kumar and Anand, 1998). In the second step, called the adhesion step, the microorganisms attach to the conditioned surface. Attachment depends on the physicochemical properties of the bacterial cell surface, nutrient availability in the surrounding medium, and the growth stage of the bacterial cells. The pH and temperature of the contact surface also influence the degree of adhesion of microorganisms (van Loosdrecht et al., 1990, Kumar and Anand, 1998; Donlan, 2002). During the third step called the microcolony formation, the bacterial cells grow and divide by using the nutrients in the conditioning film and the environment forming a layer of cells that cover the surface and produce extracellular polymeric substances (EPS). The EPS helps to anchor the cells to the surface and stabilizes the colony from the fluctuations of the environment (Kumar and Anand, 1998). During the

next step of biofilm formation, more bacterial cells continue to attach to the substratum along with continued EPS production to form multiple layers of bacterial cells called a biofilm.

The microorganisms within the biofilm are not uniformly distributed. The deepest cells within the biofilm are in a quiescent state because they can only obtain sufficient nutrients to meet maintenance requirements to sustain viability and are, therefore, not subject to competition among microorganisms (Lewis and Gattie 1990). Composition of biofilms can be heterogenous, due to the colonization of different microorganisms possessing different nutritional requirements (James et al., 1995). A biofilm can be established in as little as four hours and consists mainly of water along with up to  $10^5$  cells/cm<sup>3</sup> and extracellular polymeric substances. A mature biofilm is usually established in 24-48 h and can contain up to  $10^{10}$  cells/cm<sup>3</sup> and 20 to 30 layers of bacteria. The biofilm formation is a slow process and depending on culture conditions it takes from a few hours to several days or several months to reach a state of equilibrium (Marshall et al., 1992).

The bacteria in the biofilm are able to detach and disperse from the aging biofilm in order to survive and colonize new niches. This occurs by detachment of the daughter cells individually or by sloughing of relatively large particles of biomass of the biofilm (Kumar and Anand, 1998; Donlan, 2002).

### **EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)**

The development of extracellular matrix and growth is important for stability and maintenance of optimum environmental conditions in biofilms. Various terms such as glycocalyx, slime, capsule and sheath have been often used to refer to the EPS associated

with the biofilms (Kumar and Anand, 1998). After the initial contact with the surface, the microorganisms start producing thin fibers that become thicker with time (when viewed with a scanning electron microscope) forming a biofilm matrix called glycocalyx, which can trap many other organic and inorganic substances and particulate matter and other microorganisms (Marshall, 1992). The glycocalyx is an integral element of the outer membrane of the Gram-negative cells and the peptidoglycan of the Gram-positive cells. It is composed of either fibrous polysaccharides or globular glycoproteins and in its hydrated state consists mostly of water at about 50-95% (Kumar and Anand, 1998).

EPS constitutes 50% to 90% of the total organic carbon of biofilms and may vary in chemical and physical properties, but it is primarily composed of polysaccharides. The polysaccharides in the EPS of Gram-negative bacteria are neutral or polyanionic. The uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates present in the EPS confers the anionic property and attract divalent cations such as calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm (Sutherland, 2001). The EPS also plays a critical role in the persistence and survival in hostile environments by trapping and retaining the nutrients for the growth of biofilms and protecting the cells from the effects of antimicrobial agents (Carpentier and Cerf, 1993).

### **GENETICS OF BIOFILM FORMATION**

The genetic basis for attachment to surfaces has been studied in *E. coli* (Pratt and Kolter, 1998) and *Pseudomonas aeruginosa* (O'Toole and Kolter, 1998a). In *E. coli*, both type 1 pili and flagella are important for attachment to the surface. Some researchers observed that flagellated organisms show increased attachment compared to those

without flagella (Notermans et al., 1991) and suggested that the greater accumulation of motile organisms resulted from flagellar activity and chemotaxis. In *P. aeruginosa*, flagella are required to bring the bacterium close to the surface and the lipopolysaccharide outer membrane proteins are believed to mediate early interactions. Type IV pilus-mediated twitching motility is required for the cells to aggregate into microcolonies from a monolayer. There are also changes in gene expression such as upregulation of the alginate biosynthesis genes and downregulation of flagellar synthesis. In *P. aeruginosa* biofilm maturation also requires production of cell-to-cell signaling molecules (acyl-homoserine lactones) which accumulate and trigger the expression of specific sets of genes (Costerton et al., 1999).

### **BIOFILMS IN THE FOOD INDUSTRY**

Biofilms are a common phenomenon in the food industry and have been shown to have increased resistance to sanitizers (Kumar and Anand, 1998; Norwood and Gilmour, 2000). Biofilms formed by spoilage and pathogenic microflora in food processing environments can lead to hygienic and economic problems due to cross contamination and post-processing contamination. Cross-contamination can result in a wide range of foodborne outbreaks of pathogens such as *E. coli* O157:H7 (Beuchat 2002; Mead et al., 1999). Foodborne pathogens and spoilage microorganisms have been found to form biofilms on stainless steel, aluminium, glass, Buna-N, Teflon seals and nylon materials typically found in food-processing environments (Kumar and Anand, 1998). *E. coli* O157:H7 has been shown to form biofilms on surfaces such as stainless steel (Dewanti and Wong, 1995; Ryu et al., 2004). Cross-contamination in abattoirs (Bouvet et al., 2001; Warriner et al., 2002) and other food processing plants (Beuchat and Ryu, 1997; Warriner

et al., 2002) have been shown to be responsible for the recent outbreaks of *E. coli* O157:H7.

The material most commonly used in the food processing environment is stainless steel. The most commonly used grade is AISI (American Iron and Steel Institute Standards) type 304 made mostly of iron, with 18% chromium and 9% nickel. Stainless steels have an oxide film on the surface, composed of oxy-hydroxides of chromium and iron and that makes the steel resistant to corrosion from acidic or neutral chlorinated solutions. Adhesion of microorganisms to chemically inert solid surfaces such as stainless steel is a physico-chemical phenomenon, resulting from electrostatic and nonelectrostatic interactions between the surface of the solid and the bacterium and depend on the properties of the surface, the bacterium and the surrounding liquid medium (Boulangé-Petermann, 1996).

### **FACTORS AFFECTING BACTERIAL ATTACHMENT**

The adhesion behavior of microorganisms is governed by their surface properties such as hydrophobicity which specifically depends on the presence and absence of cell surface appendages which are also controlled by the culture method. The pH of the suspension medium can induce a change in the surface charge of the bacterium and consequently increase or reduce adhesion (Boulangé-Petermann, 1996; Notermans et al., 1991).

Microbial adhesion is also affected by the temperature of the medium (Stanley, 1983; Herald and Zottola, 1989). It was shown that at low temperatures, the adhesion and subsequent production of biomass in the biofilm are time-dependent and growth of most microorganisms is delayed because of the effects of temperature on chemical and

physical adsorption processes, medium viscosity and microbial physiology (Notermans et al., 1991). Czechowski (1990) studied the adhesion of bacteria to various inert substrates and found that adhesion followed a biphasic pattern on stainless steel at all temperatures used (5°C, 11°C, and 25°C), but on synthetic rubber Buna-N and on Teflon adsorption was linear with time at 5°C and 25°C, showing the importance of the nature of the substrate on adsorption kinetics at different temperatures. Ryu and Beuchat (2004) showed that low nutrient availability and low incubation temperatures (12°C versus 22°C) favors the production of EPS by *E. coli* O157:H7.

The time of contact between the bacterial and solid surfaces also plays an important role in the adhesion process. As the contact time increases, the number of microorganisms adhering to the surface also increases, because the probability of a cell colliding with the surface increases with time (Notermans et al., 1991).

### **CLEANING AND DISINFECTION**

Disinfection can be defined as the elimination of disease-causing microorganisms. The disinfectants used in the food industrial environments must be effective, safe, easy to use, and not affect the sensory qualities of the food product. A 3-log reduction (99.9%) has been recommended as a target for effective inactivation of biofilm bacteria (Frank and Chmielewski, 1997; Wirtanen et al., 2001). In food processing plants, the sanitation process usually consists of the application of a cleaning product and water rinse, followed by a sanitizer. Alkaline or acid detergents are generally used to clean food processing equipment. Alkaline detergents have a powerful saponification capacity while acid detergents are able to dissolve mineral deposits. Although some of these disinfectants are inexpensive, they can cause pollution problems if they are not treated before release into

the environment. Inappropriate use of acids can also lead to corrosion of stainless steel. After each cleaning and disinfecting operation, the surfaces must be rinsed well to avoid contamination of the food product by residues of the cleaner or disinfectant ((Boulange-Petermann, 1996). Quaternary ammonium chlorides (QAC) are a class of hydrophilic cationic compounds that are used to disinfect surfaces. Since the bacterial surface is hydrophilic and negatively charged, QAC readily adsorb to it, penetrate the cell wall, and disrupt the cytoplasmic membrane (Boulange-Petermann, 1996).

### **RESISTANCE OF BIOFILMS TO ANTIMICROBIALS**

Bacterial biofilms have been shown to have increased resistance to antimicrobial treatments than planktonic cells in suspension. The properties associated with biofilms such as reduced diffusion, physiological changes due to reduced growth rates and the production of antimicrobial degrading enzymes make the biofilms resistant to antimicrobials (Frank and Koffi, 1990; Krysinski et al., 1992). The EPS matrix surrounding the biofilms is believed to act as a diffusion barrier, a molecular sieve and an adsorbent that protects the cells by binding with antimicrobial substances and quenching their effect as they diffuse through it (Boyd and Chakrabarty, 1995).

Efficacy of a disinfectant depends on the type and the characteristics of the surface material used such as chemical composition, superficial charge, hydrophobicity, roughness (Donlan, 2002). The penetration of biocides into biofilm depends on their effective concentration at the biofilm surface and time of contact of biocide with the biofilm (Tashiro et al., 1991). The susceptibility of bacteria to antimicrobial agents is also greatly influenced by conditions used in the cultivation of the organisms. The conditions

of use such as temperature and pH may also influence the efficacy of antimicrobials (Anwar et al., 1990).

### **CETYLPYRIDINIUM CHLORIDE (CPC)**

Cetylpyridinium chloride, or 1-hexa-decyl pyridinium chloride, is a quaternary ammonium compound with antimicrobial properties against many microorganisms including viruses (FDA, 1998). It is a cationic surface-active agent with a cetyl radical substituted for hydrogen atom on position 1. Research has indicated that 0.05% to 0.5% CPC in mouthwashes reduces or inhibits bacterial gingivitis, biofilm, or plaque formation (Wilson et al., 1996; Renton-Harper et al., 1996). It is currently used in commercial mouthwashes at a maximum permitted concentration of 0.1% by the U.S. Food and Drug Administration. Currently, it is only approved for use in poultry processing. The LD50 for rats is 20 mg/kg when administered orally as a pure compound (FDA, 1998).

The antimicrobial activity of CPC is because of the charged cetyl radical that reacts with the cell membrane of the bacterial cell possessing a net negative charge. This results in the leakage of the cellular components, disruption of cell metabolism, prevention of growth and replication, and cell death. CPC is not corrosive to metal, does not add to phosphate waste, and is not a severe health hazard (FDA, 1998).

In order to study bacterial attachment and colonization, a variety of experimental methods have been developed. Sonication followed by standard plate counts are indirect methods that first detach the microorganisms from the surface and then count them. According to Lindsay and von Holy (1999), sonication, vortexing, and shaking with beads were equivalent methods, based on bacterial counts. Other indirect methods such as radiolabeled bacteria, enzyme-linked immunosorbent assay, biologic assays, stained

bacterial films, and microtiter plate procedures estimate the number of attached organisms in situ by measuring some attribute for the attached organism. Methods involving direct observation, including microscopy techniques (light, laser-scanning confocal, transmission electron, and scanning electron microscopy), are used to observe biofilms directly (Costerton et al., 1995).

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## CHAPTER 3

### DETECTION OF *ESCHERICHIA COLI* O157:H7 IN CATTLE WATER TROUGHS OF FOUR CATTLE FARMS IN LOUISIANA

## INTRODUCTION

*Escherichia coli* O157:H7 is an emerging foodborne pathogen that causes an estimated 73,000 illnesses, leading to 2,000 hospitalizations, 60 deaths and most cases of the hemolytic uremic syndrome in the United States each year (Rangel et al., 2005). *E. coli* O157:H7 is characterized by the presence of Shiga toxin (*stx*) genes, the *eae* gene coding for adhesion protein, intimin, and a large molecular weight plasmid that contains the gene *hlyA* encoding for a hemolysin (Tarr, 1995). Cattle are considered to be the major reservoir for *E. coli* O157:H7. Several outbreaks of *E. coli* O157:H7 infections have been related to eating undercooked ground beef, visiting farms and handling animals (Sanchez, 2002). Cattle feces are the principal source of the *E. coli* O157:H7 and most human outbreaks have occurred from the consumption of foods contaminated directly or indirectly with bovine feces (Armstrong et al., 1996). Studies have shown that the organism can persist in cattle feces, cattle feed, and water troughs on farms (Hancock et al., 1998; Rice et al., 1999; Shere et al., 1998). It has been observed that human infection with *E. coli* O157:H7 is more common in the warm summer months than in the winter months (Rangel et al., 2002). Other studies have shown that cattle also shed this pathogen in the summer, suggesting that factors such as temperature play an important part in determining the frequency of human infections (Elder et al., 2003).

*E. coli* O157:H7 is widely distributed in cattle populations throughout the world. In the United States, *E. coli* O157:H7 is prevalent in individual cattle from 0 to 28%, whereas the herd prevalence varies from 0 to 75% (Sanchez et al., 2002). Contaminated water troughs can provide an environment niche for survival, proliferation, and horizontal spread of *E. coli* O157:H7 on cattle farms (Maule, 2000; LeJeune et al., 2001b). Feed and

drinking water offered to livestock may contribute to the prevalence of infection in cattle and subsequent contamination of beef products during slaughter (LeJeune, 2001b). *E. coli* have been shown to survive, persist and proliferate in other aquatic environments suggesting that they can also survive within the water troughs and be a source of continuous infection to cattle (Davies et al., 1995). Although cattle are considered to be the primary reservoir for this pathogen, fecal excretion of *E. coli* O157 by cattle occurs only occasionally and lasting 3 to 4 weeks. However, *E. coli* O157 is found to persist in the farm environment for long periods of time, from several months to several years (Hancock et al., 1997; Shere et al., 1998). The high occurrence of O157:H7 in cattle water troughs suggests that the sediments and the biofilms in water troughs may serve an environmental reservoir for this organism (Hancock et al., 1997).

Previous studies conducted by our laboratory on one of the cattle farms in Louisiana found an *E. coli* O157:H7 prevalence of 4.9% (8 positive out of 164 cattle tested) in the cattle. Based on these findings it was hypothesized that the water troughs in the cattle farms may possibly be contaminated with *E. coli* O157:H7. The objectives of this study were (i) to examine the prevalence of *E. coli* O157:H7 in cattle water troughs in four cattle farms in Louisiana (ii) to characterize the isolates by determination of *stx1*, *stx2*, *eaeA*, *hlyA* genes and RAPD (Random amplification of polymorphic DNA) patterns, (iii) to determine the antibiotic susceptibilities and cytotoxicity of the isolates.

## **MATERIALS AND METHODS**

### **Sample Collection**

A total of four farms were selected from 4 locations in Louisiana: the northwest, the west, the southeast and the central locations. Samples were collected one time during September and October of 2005 from each of the four farms. From each selected water

trough, water and sediment samples and also algal biofilms on the sides and bottom of the trough were sampled. Samples were collected using aseptic techniques into sterile containers and were transported to the lab, stored overnight at 4°C, and analyzed the next day. A total of 40-60 samples/farm were collected for the entire study.

### **Recovery of *E. coli* O157 from Water Trough Samples**

Water trough samples: For each sample, 30 ml of trough water were transferred into a sterile specimen cup and combined with 30 ml of a 2 x concentrate of TSB, mixed and incubated overnight at 37°C for 18-24 h. Trough biofilm and sediment samples: For each sample, 20 to 50 ml of a 2 x concentrate of TSB (dependent on the volume of the sample) were added after which the suspension was briefly shaken and then incubated overnight at 37°C for 18-24 h. After incubation, immunomagnetic separation (Dynabeads *E. coli* O157, Dynal Inc., Oslo, Norway) was performed for each sample.

### **Isolation of *E. coli* O157:H7 Using Immunomagnetic Separation (IMS)**

One milliliter portions of the enriched homogenate were mixed with 20 µl supermagnetic polystyrene beads coated with affinity purified *E. coli* O157 antibodies (Dynal Biotech, Norway). Separation and washing procedures were followed according to the manufacturer's instructions. Washed beads were resuspended in 100 µl wash buffer (PBS Tween: 0.15 M NaCl, 0.01 M Sodium-Phosphate buffer, pH 7.4, with 0.05 % Tween-20) and 50 µl were streaked on two Sorbitol MacConkey agar plates containing 2.5 µg of potassium tellurite/ml and 50 ng of cefixime/ml (SMAC- CT). The SMAC-CT plates were incubated at 37°C for 18-24 h. Following incubation, colonies exhibiting morphology typical of presumptive sorbitol negative *E. coli* O157 (gray or pale with a darker center) were tested for indole (addition of 0.2-0.3 ml Kovacs' reagent to 5 ml of

24 h tryptophane broth inoculated with the test bacteria). All SMAC colonies that were found to be sorbitol negative and indole positive (dark pink color of tryptophane broth) were checked for agglutination with O157 and flagellar H7 antiserum according to the manufacturer's recommendations (Remel Inc., Lenexa, USA). Morphologically typical, indole-positive, non-sorbitol-fermenting colonies which were positive for O157 and H7 latex agglutination were considered presumptive *E. coli* O157:H7 based on culture and latex agglutination. These isolates were then tested for the presence of virulence genes by PCR (Polymerase Chain Reaction).

#### **PCR for *stx1*, *stx2*, *eaeA*, and *hlyA*, 16S rRNA, O157 and the Flagellar H7 Gene Amplification**

Three sets of multiplex PCRs for the detection of *stx1* and *stx2*, *eaeA* and *hlyA*, O157 and the flagellar H7 genes, and a PCR for 16S rRNA were performed by a GeneAmp PCR thermocycler (Model 2400, Perkin-Elmer, USA). Oligonucleotide primers for all the genes were purchased from Integrated DNA Technologies, Inc., Coralville, Iowa. Oligonucleotide sequence of primers (Osek, 2003) and the predicted sizes of PCR amplified products are listed in Table 1. Crude template DNA for each isolate was prepared by adding 25 µl of an overnight culture to 500 µl of sterile distilled water and boiling at 95°C for 5 min.

The PCR assays was performed in 50 µl reaction volume containing 25 µl Taq PCR Master Mix (premixed solution containing 5 units/µl *Taq* DNA Polymerase, PCR buffer containing both KCl and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub> and 200 µM each dNTP; Qiagen Inc., USA), 3 µl (0.5 µM) of primer, 14 µl of Millipore water, and 5 µl of template DNA. The PCR thermocycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for

**Table 1: PCR primers used in this study (Osek, 2003)**

Primer set	Target gene	Sequence (5'→3')	Location within gene	Size of PCR product (bp)
A	<i>stx1</i>	CAGTTAATGTCGTGGCGAAGG CACCAGACAATGTAACCGCTG	213-232 559-538	348
	<i>stx2</i>	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	295-316 881-859	584
B	<i>E.coli</i> 16S rRNA	AGAGTTTGATCATGGCTCAG GGACTACCAGGGTATCTAAT	8-27 805-798	798
C	<i>eaeA</i>	GGGATCGATTACCGTCAT TTTATCAGCCTTAATCTC	26010-26027 26847-26832	837
	<i>hlyA</i>	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	70-906 602-578	534
D	<i>rfb</i> O157	CGTGATGATGTTGAGTTG AGATTGGTTGGCATTACTG	918-935 1339-1321	420
	<i>fliC</i> H7	GCTGCAACGGTAAGTGAT GGCAGCAAGCGGGTTGGT	553-570 1500-1483	948

1 min. The final extension step was at 53°C for 5 min (Osek, 2003). The amplified PCR products were separated on 1.0% agarose gels in TBE (Tris-Borate-EDTA) buffer, followed by staining with SYBR® Gold nucleic acid gel stain (Molecular Probes, Invitrogen, USA) and photographed under UV illumination.

### **Vero Cell Cytotoxic Assay**

After confirmation by multiplex PCR, the Vero cell cytotoxic assay was performed to characterize all the *E. coli* O157:H7 isolates in this study. A continuous African green monkey kidney (Vero) cell line was maintained by weekly trypsinization of confluent monolayers grown in Eagles's Minimum Essential Medium (EMEM; ATCC, USA) containing 10% fetal calf serum. The Vero cell assay was performed on aliquots of filter-sterilized mitomycin C treated enrichment broth after centrifugation.

Fresh monolayers of Vero cells (ATCC, USA) were prepared by suspending 200 µl of  $10^5$  cells/ml in 96-well polystyrene cell culture plates. The cells were allowed to grow for 2 days, at which time fresh growth medium (200 µl per well) was added. Culture filtrate (50 µl) of individual isolates was added to duplicate wells with confluent monolayers. Culture filtrate was obtained by growing each isolate in TSB (Tryptic Soy Broth) at 37°C for 24 h, removing the cells by centrifugation (7000 x g, 10 min), and filtering the supernatant fluid through a 0.2 µm-pore size membrane filter. Plates were sealed, incubated in a carbon dioxide enriched atmosphere (5%, w/v) at 37°C, and then examined under an inverted microscope after 48 h. In the presence of shiga toxins, the Vero cell monolayer is disrupted and cells become detached. Isolates were considered to be positive for toxin production when 50% of the Vero cells were dead and detached.

#### **Antimicrobial Susceptibility Test**

The antimicrobial susceptibility of the *E. coli* O157:H7 isolates was determined using the VITEK Gram-Negative Susceptibility (GNS) cards of the VITEK system (BioMérieux, France). The principles of the GNS card are based on the microdilution minimum inhibitory concentration (MIC) technique. The card is a miniaturized and abbreviated version of the doubling dilution technique (VITEK Microbiology Reference Manual). The GNS card consists of 45 wells with each well containing aliquots of individually weighed, pre-measured portions of a specific antimicrobial agent combined with microbiological culture media. After inoculation of the sample, the card was placed inside the VITEK Reader which measures the presence or absence of growth in each well according to light attenuation which is measured by an optical scanner. The results were obtained as MIC values which were determined by NCCLS (National Committee for

Clinical Laboratory Standards) reference methods. A total of 17 antimicrobials tested were: amikacin, amoxicillin/clavulanic acid, ampicillin, carbenicillin, ceftazidime, ceftiofur, cephalothin, chloramphenicol, ciprofloxacin, enrofloxacin, gentamicin, nitrofurantoin, piperacillin, tetracycline, ticarcillin, tobramycin and trimethoprim/sulfamethoxazole. The results were interpreted by NCCLS guidelines.

### **RAPD of Chromosomal DNA**

Primers for RAPD were purchased from Integrated DNA Technologies, Inc., Coralville, Iowa. The 10-mer primers used were 1254 (5' CCG CAG CCA A 3') and 1283 (5' GCG ATC CCC A 3'). Chromosomal DNA was extracted by adding 500 µl of an overnight culture to 500 µl of sterile distilled water and boiling at 95°C for 5 min. The PCR assay was performed in 50 µl reaction volume containing 25 µl *Taq* PCR Master Mix (premixed solution containing 5 units/µl *Taq* DNA Polymerase, PCR buffer containing both KCl and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub> and 200 µM each dNTP; Qiagen Inc., USA), 3 µl (0.5 µM) of primer, 14 µl of Millipore water, and 5 µl of template DNA in a total reaction volume of 50 ml. Amplification was done with a thermal cycler which was set for initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 20 sec, 45°C for 30 sec, and 72°C for 1 min; followed by 1 cycle at 72°C for 10 min. After the reaction, the amplified DNA was electrophoresed on a 1.5% agarose gel for 90 min. After electrophoresis, the gel was stained with SYBR® Gold nucleic acid gel stain (Molecular Probes, Invitrogen, USA) and photographed under UV illumination.

## **RESULTS**

Previous studies conducted by our laboratory on a cattle farm in Louisiana confirmed that cow feces were potential sources of *E. coli* O157:H7 (Refer to page 36).

Fecal samples from 110 cows and 54 calves showed a prevalence for *E. coli* O157:H7 of 4.9% (8 positive out of 164 cattle tested) in the cattle. In this study, cattle water troughs were tested for the presence of *E. coli* O157:H7. A total of four farms were selected from four different locations in Louisiana, one of which was also used in the previous study to determine *E. coli* O157:H7 prevalence in cattle. However, of the four farms investigated, only two farms one of which was used in the previous study from our laboratory were positive for *E. coli* O157:H7 in cattle water troughs.

Out of the 176 total samples collected from the four farms, 8 (4.5%) samples were positive for *E. coli* O157:H7. Of the 176 samples, 82 were water samples and 96 were biofilm/sediment samples, of which two (2.4%) water and six (6.4%) biofilm/sediment samples yielded *E. coli* O157:H7. *E. coli* O157:H7 was isolated from 2 biofilm/sediment and 2 water samples from one of the farms and 4 biofilm/sediment samples from another farm. Among the 76 matched sets of water and biofilm and sediment samples from the same troughs, none yielded *E. coli* O157:H7 in both samples.

PCR amplification of the *stx* genes showed that all the 8 isolates possessed the *stx2* gene but not the *stx1* gene (Fig 3.1 A). All the 8 isolates were positive for the virulence plasmid encoding the enterohemolysin gene but were negative for the intimin-coding *eae* gene (Fig. 3.2. C). The presence of O157 and H7 antigens was also confirmed by PCR (Fig. 3.2 B). Amplification of 16S rRNA was used to confirm that the isolates were *E. coli*. The Vero cell assay was used as a confirmation test to determine the virulence potential of *E. coli* O157:H7 isolates. All the isolates produced Stx, as assessed by the Vero cell cytotoxicity assay. Fig. 3.3 shows the cytopathic effects of verotoxin preparations on Vero cells. Microscopic analysis of Vero cells after exposure to toxin

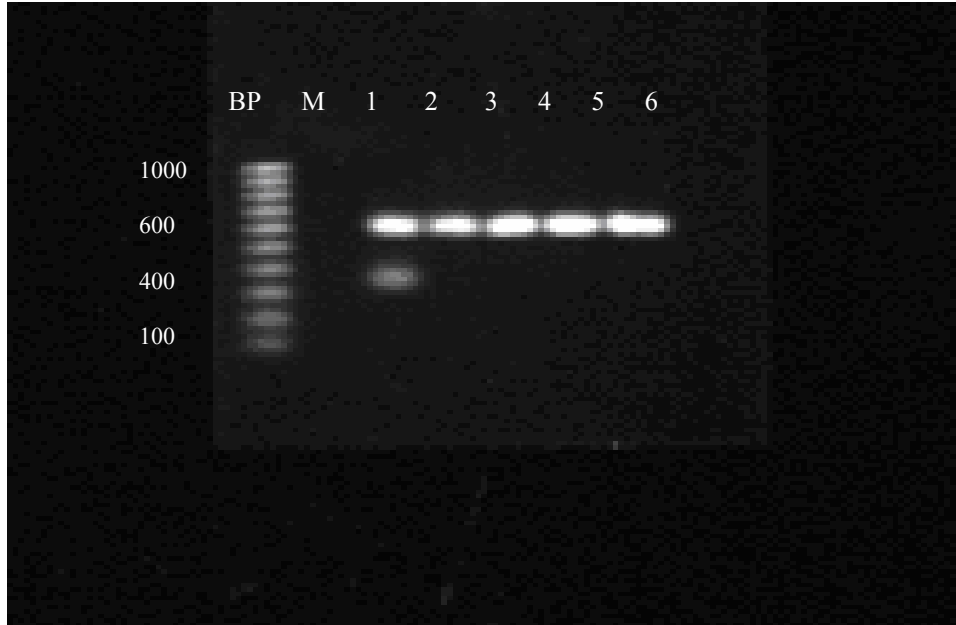
preparations showed extensive cell damage, detachment, vacuole formations, and death. Antibiotic susceptibility tests: All the *E. coli* O157:H7 isolates were susceptible to all the 17 test antibiotics tested.

## DISCUSSION

The beef cattle industry is the second largest animal production industry in Louisiana. In 2003, the number of beef cattle producers was 12,522 with a gross farm income of \$291.8 million. Beef production is statewide with 63 of 64 parishes generating income from beef production (LSU AgCenter). There have been very few studies on the prevalence of *E. coli* O157:H7 in Louisiana cattle farms. In one of the few studies reported in the literature on the prevalence of *E. coli* O157:H7 in Louisiana, Dunn et al. (2004) found that 38.5% of herds and 6.5% of animals in dairy herds were positive for *E. coli* O157:H7 during the summer. In another study by the same authors in Louisiana, *E. coli* O157:H7 was found in deer; however, it was found that deer were not a significant reservoir for *E. coli* O157, since only 0.3% of hunter-harvested deer tested positive. Human infections with *E. coli* O157:H7 became reportable in Louisiana in 1996. The number of cases ranges from five to twenty-five per year and has remained stable every year. Fig. 3.4 shows *E. coli* O157:H7 average annual cases by month in Louisiana (Louisiana Office of Public Health).

Fecal shedding of *E. coli* O157:H7 by cattle occurs most frequently in the warmer months (Hancock et al., 1997; Mechie et al. 1997). Infection with *E. coli* O157:H7 in humans is also more common in summer and fall suggesting that climactic factors play an important part in determining the incidence of human infections (Tarr et al., 2005).

A



**Fig. 3.1. Results of PCR for detection of *stx1*, *stx2*, *eaeA*, *hlyA*, O157 and flagellar H7 genes.**

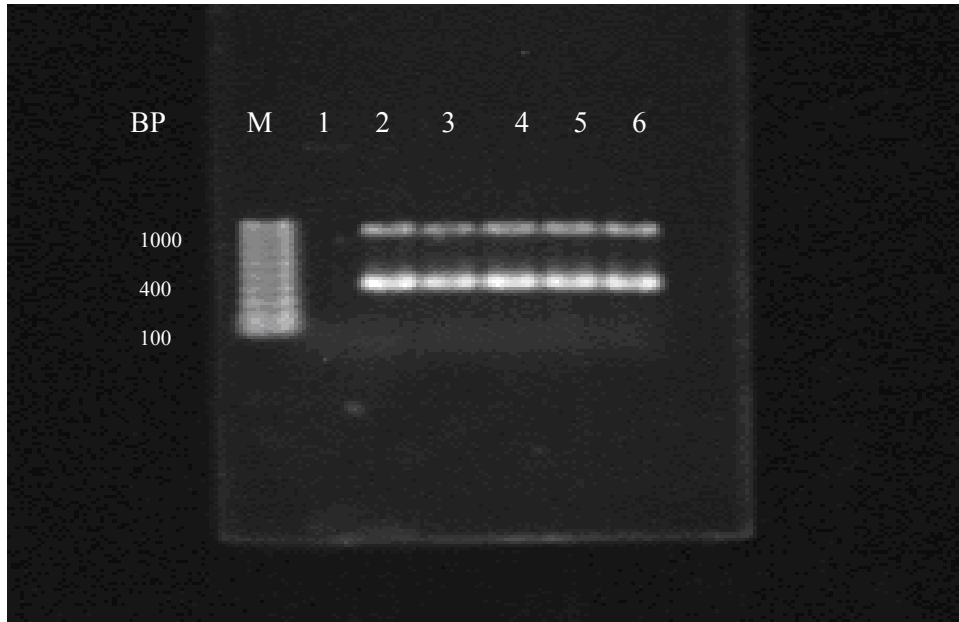
(A) PCR for detection of *stx1* and *stx2* toxin genes in the *E. coli* O157:H7 isolates. PCR products (548 bp) were separated in a 0.8% agarose gel. Lane M contains 100 bp DNA size marker. Lane 1 is the negative control (non-157:H7 *E. coli*) and lane 2 is a positive control (*E. coli* O157:H7 with *stx1* and *stx2* genes). Lanes 3, 4, 5 and 6 lanes are the *E. coli* O157:H7 isolates from the cattle water troughs. All the isolates were positive for *stx2* (548 bp) but were negative for *stx1* (348 bp).

(B) PCR for detection of O157 and H7 antigens of *E. coli* O157:H7 isolates (PCR products 420 bp and 948 bp respectively). Lane M contains 100 bp DNA size marker. Lane 1 is the negative control (non-157:H7 *E. coli*) and lane 2 is a positive control (*E. coli* O157:H7). Lanes 3, 4, 5 and 6 lanes are the *E. coli* O157:H7 isolates from the cattle water troughs. All the isolates were positive for the O157 and H7 antigens.

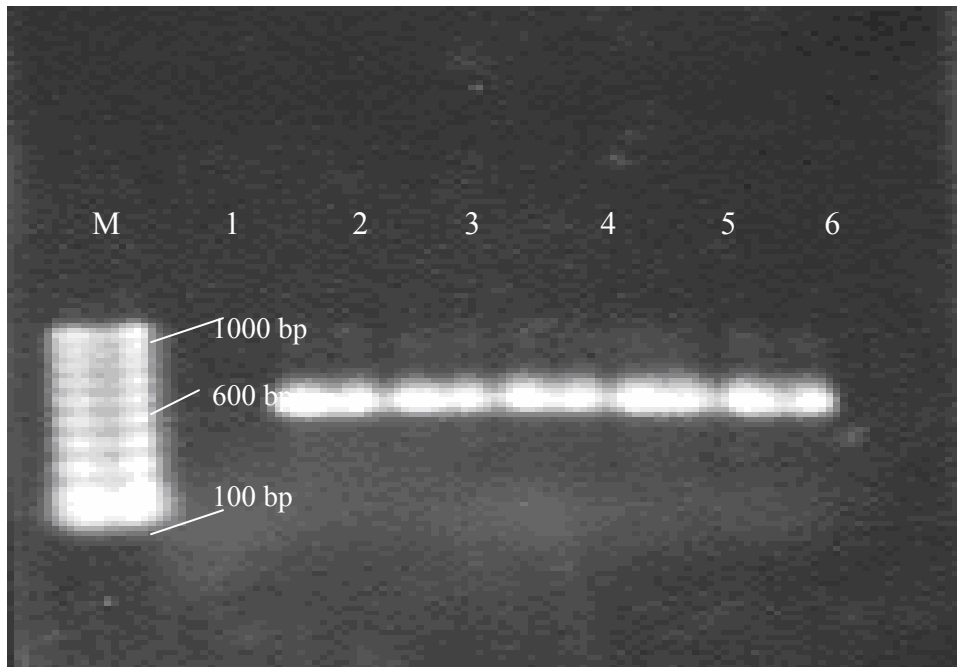
(C) PCR for detection of intimin and enterohemolysin genes of *E. coli* O157:H7 isolates (PCR products 837 bp and 534 bp respectively). Lane M contains 100 bp DNA size marker. Lane 1 is the negative control (non-157:H7 *E. coli*) and lane 2 is a positive control (*E. coli* O157:H7). Lanes 3, 4, 5 and 6 lanes are the *E. coli* O157:H7 isolates from the cattle water troughs. All the isolates were positive for the enterohemolysin gene but negative for the intimin gene.

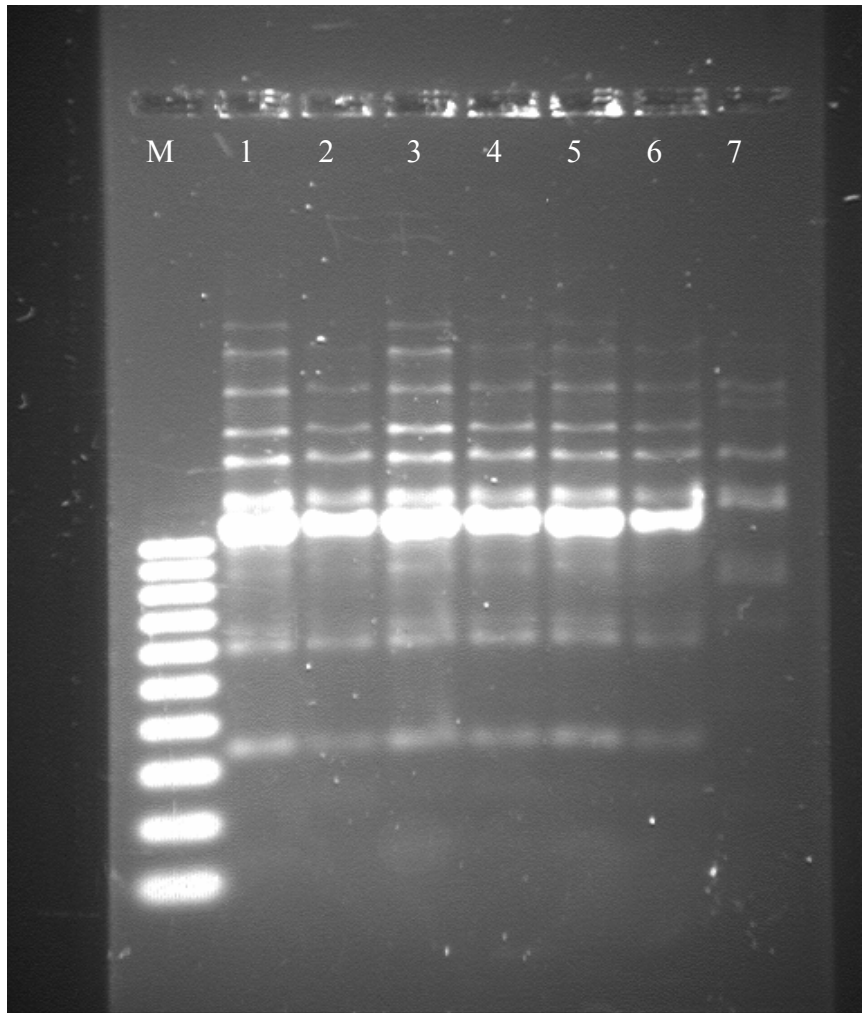
Fig. 3.1 (continued)

**B**



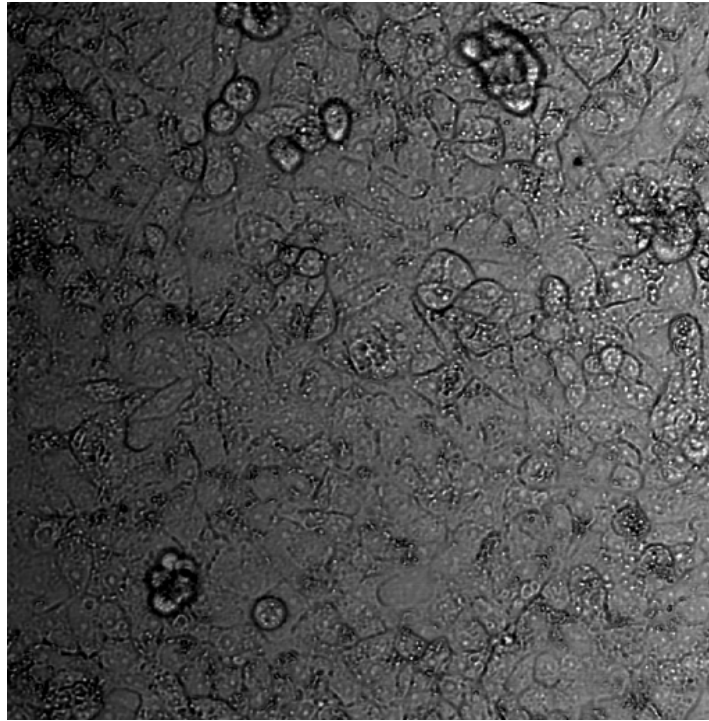
**C**



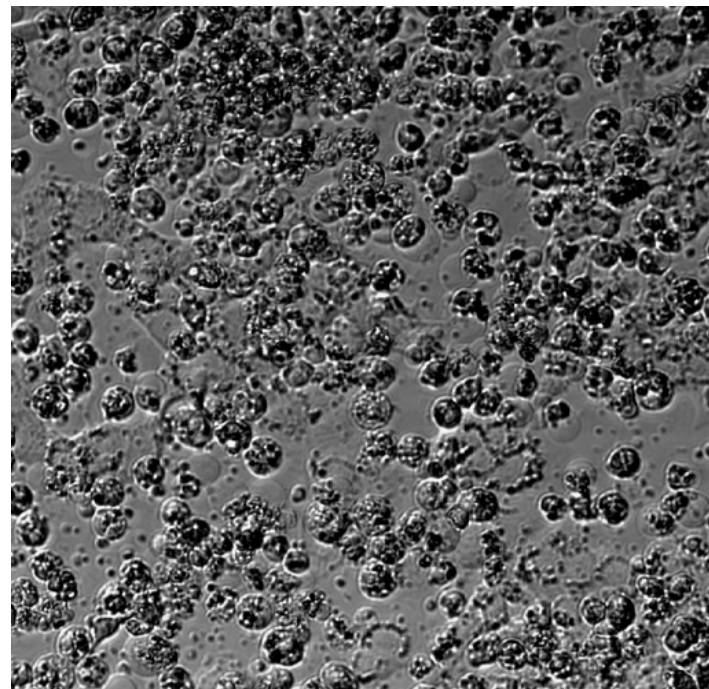


**Fig.3.2. RAPD patterns of *E. coli* O157:H7 isolates.** Lane M is the 100 bp DNA marker; lanes 1 to 6 are the *E. coli* O157:H7 isolates obtained in this study; lane 7 is non-O157:H7 *E. coli*.

A



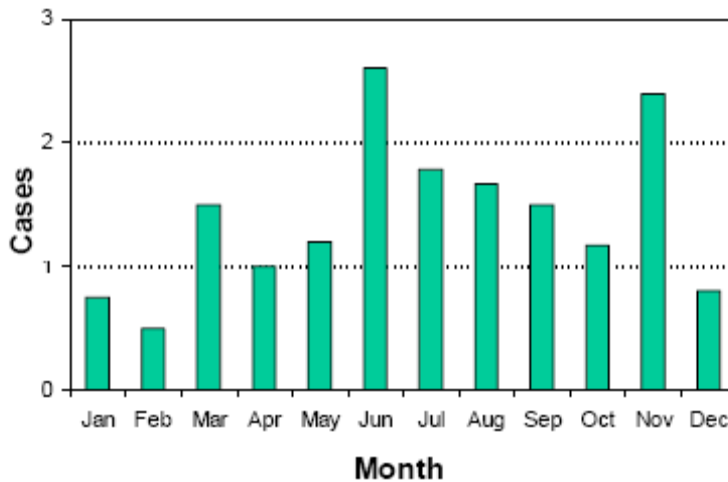
B



**Fig. 3.3. Results of Vero cell cytotoxic assay.**

(A) control Vero cells (B) Vero cells treated with the culture filtrate of one of the *E. coli* O157:H7 isolates from this study.

Therefore, samples for this study were collected during September and October of 2005 to increase the chances of detecting *E. coli* O157.



**Fig. 3.4. *Escherichia. coli* O157:H7 average annual cases by seasonal distribution - Louisiana, 1996-2004.** (Source: Louisiana Office of Public Health – Infectious Disease Epidemiology Section – Annual Report, 2004)

We found a 4.5% prevalence of *E. coli* O157:H7 in the cattle water troughs of the four farms tested. Water troughs on farms have commonly been found to contain *E. coli* O157:H7 (Faith *et al.* 1996; Shere *et al.* 1998, McGee *et al.*, 2002). Contaminated water troughs are shared by all animals within a group/herd and can provide an environment for survival, proliferation, and an important source of *E. coli* O157:H7 infection for animals. Several studies have demonstrated that contaminated water troughs can play a role in waterborne transmission of *E. coli* O157:H7 to cattle (LeJeune *et al.*, 2001a; Shere *et al.*, 2002). LeJeune *et al.* (2001a) showed that *E. coli* O157:H7 which had survived for more than 6 months in water trough sediments was infectious to calves. In a study of 2 feedlots by Van Donkersgoed (2001), the prevalence rate of *E. coli* O157:H7 in water troughs was found to be 12%. LeJeune *et al.* (2004), in a study of commercial feedlots, found that the prevalence rate of *E. coli* O157:H7 in water trough samples was as high as 26% (22 of

86) and 17% (15 of 86) in water troughs supplied with unchlorinated and chlorinated water, respectively. The *E. coli* O157:H7 isolated from feces, water troughs, and other places in the farm, in both these studies, shared some common subtypes suggesting transmission of *E. coli* O157:H7 among these sites.

In a study of feedlot cattle in four states by Sargeant et al. (2003), the prevalence of *E. coli* O157:H7 in the water troughs was found to be 13.1% and 60.3% of feedlots had at least one positive water tank. They also found that the cattle were more likely to be shedding *E. coli* O157:H7 in pens with positive water tanks, and water was more likely to be positive when *E. coli* O157:H7 was detected in the sediments of the water tanks. Although *E. coli* O157:H7 is by shed cattle only transiently (Besser et al., 1997; Hancock et al., 1997), it can persist in the farm environments for up to 2 years (Mechie et al., 1997). Several studies have shown that the organism can survive in the water troughs for extended periods of time (Hancock et al., 1998; Shere et al., 1998; Kudva *et al.*, 1998; Rice and Johnson, 2000).

The detection of *E. coli* O157:H7 in water troughs in this study and several others suggest that the sediments and biofilms formed in the water troughs could serve as an environmental reservoir for this pathogen. Water troughs can become contaminated with *E. coli* O157:H7 from cattle feed, feces or oral secretions. It was noted in our study that the water troughs from which *E. coli* O157:H7 was recovered during this study were in highly unsanitary conditions with large amounts of sediments composed of soil, feed and biofilms at the bottom of the water troughs. Similar observations were made by Hancock et al. (1998) who found that *E. coli* O157:H7 was not recovered from water troughs that were frequently cleaned during the study period. However, some studies have shown that

there was no relationship between effect of trough cleaning on prevalence of *E. coli* O157:H7 (Sargeant et al., 2004; LeJeune et al. 2001b).

It has been shown that human isolates of *E. coli* O157:H7 that have both *stx1* and *stx2*, and those that have only *stx2* are very common, but isolates having only *stx1* are uncommon (Ostroff, et al., 1989). All of the *E. coli* O157:H7 organisms isolated in this study were *stx1* negative and *stx2* positive and thus potentially pathogenic to humans. Intimin is an outer membrane protein encoded by the chromosomal *eae* gene that acts as an adhesin and occurs in most *E. coli* O157:H7, but not all *E. coli* O157:H7 strains (Donnenberg et al., 1993). In addition, most *E. coli* O157:H7 strains carry a ~90 kilobase plasmid which encodes production of a hemolysin, also known as enterohemolysin. All of the isolates in this study were negative for the *eae* gene but possessed the enterohemolysin gene.

Each bacterial isolate was tested for susceptibility to 17 antimicrobial agents. All the *E. coli* O157:H7 isolates were susceptible to all the test antibiotics. Antibiotics are normally used in cattle feedlots for therapeutic, disease prophylaxes, and growth promotion and it is believed that agricultural use of antibiotics accounts for the majority of increases in antibiotic-resistant human isolates. It is generally believed that antimicrobial resistance in *E. coli* O157:H7 is low (0.8 to 8%) and limited to a few antimicrobials, tetracycline, streptomycin, sulfamethoxazole, and trimethoprim (NARMS) when compared with other foodborne pathogens (Sanchez et al., 2002). However, resistance to multiple classes of antimicrobials has been described in *E. coli* O157:H7 and there is evidence showing that antibiotic resistance in *E. coli* O157:H7 is increasing (Galland et al., 2001; Kim et al., 1994; Meng et al., 1998; Mora et al., 2005;

Schroeder et al., 2002; Zhao et al., 2001). Since most human *E. coli* O157:H7 infections are related to eating contaminated beef products, it is important to determine if the organism develops resistance to antibiotics during food animal production in the cattle farm environments.

A number of strain differentiation methods, including Shiga toxin genotyping, plasmid profile analysis, restriction enzyme digestion and electrophoresis, multilocus enzyme electrophoretic typing, pulse field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD) and phage typing are used to characterize strains of *E. coli* O157:H7. RAPD fingerprinting was used in our study to determine molecular relatedness because it is less costly and it is faster than other methods. It has been used for strain differentiation of *E. coli* O157:H7 in many studies (Galland et al., 2001; Kim et al., 2005). Kim et al. (2005) found that RAPD could be used to distinguish strains with different virulence factors from different sources, but could not discriminate among isolates possessing only *stx1* or *stx2* and strains possessing both *stx1* and *stx2*. All the isolates obtained in our study from the two farms in two different geographic locations in the state produced identical RAPD patterns (Fig. 3.2). Identical RAPD patterns could suggest that the strains are genetically related and have a common clonal origin, but it is also possible that RAPD could not differentiate between the genetically different strains. Studies have shown that identical strains of *E. coli* O157:H7 exist in cattle herds separated by several hundred kilometers (Davis et al., 2003; Rice et al., 1999). It is believed that *E. coli* O157:H7 is transported geographically by migratory wildlife because it has also been isolated from many species of domestic and wild

animals, including cattle, sheep, goats, pigs, horses, dogs, deer, birds, wild rabbits, rats, migratory birds and gulls (Wallace et al., 1997; Makino et al., 2000).

Studies have shown that there is significant genomic diversity and great variability in virulence among *E. coli* O157:H7 isolates that have the same known virulence determinants (Baker et al., 1997). It has been shown that PFGE has greater discriminatory power than other fingerprinting methods (Pradel et al., 2001). It is possible that strain variability was missed by the less sensitive RAPD method and, therefore, further analysis with PFGE would be required to determine genetic relatedness between the isolates.

The one-time sampling of the present study does not allow the evaluation of persistence, seasonal variation, or geographical differences in prevalence of *E. coli* O157:H7 in the cattle farms examined in this study. Temporal studies may be important in evaluating any potential relationship between *E. coli* O157 persistence in the cattle water troughs and in the cattle feces. Further characterization and comparisons of isolates may be necessary to determine if the strains obtained from this study are similar to those obtained from cattle feces.

Sanitary practices on the farm are believed to play an important role in the spread of *E. coli* O157:H7 infections in a cattle farm environment (LeJeune et al., 2001b; Rice et al., 2000; McGee et al., 2002). Although cause and effect relationships between specific management practice and increased fecal shedding have not been established, improved overall sanitation on the farm to reduce fecal contamination of water troughs could reduce environmental contamination to some extent.

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## CHAPTER 4

### SURVIVAL AND GROWTH OF DIFFERENT STRAINS OF *ESCHERICHIA COLI* O157:H7 IN CATTLE WATER TROUGHS

## INTRODUCTION

It is estimated that 73,000 illnesses occur each year due to *Escherichia coli* O157:H7 infection in the United States leading to 2,000 hospitalizations and 60 deaths every year (Rangel et al., 2005). Illnesses associated with *E. coli* O157:H7 include hemorrhagic colitis, hemolytic uremic syndrome and thrombocytopenic purpura. Cattle are considered to be the main reservoir of *E. coli* O157:H7. Water troughs are a major source of exposure of cattle to enteric bacteria, including a number of foodborne pathogens. Cattle water troughs contaminated with *E. coli* O157:H7 could serve as a common source of exposure that could result in infection of large numbers of animals during a relatively brief period. It is well known that *E. coli* and *Salmonella* sp. are able to survive in other aquatic environments indicating that these bacteria can also persist and possibly proliferate as endogenous flora within the water trough environments (Burton et al., 1987; Davies et al., 1995).

Bacterial contaminants in cattle water troughs may arise from multiple sources. Cattle may contaminate the troughs with cud or fecal material. Extraneous matter including dust, feed, or bedding may also enter the trough. It is a well known fact that infection of cattle and sheep with *E. coli* O157:H7 follows a seasonal pattern, with the highest incidence of *E. coli* O157:H7 positive animals occurring in the warmer months (Hancock et al., 1994; Kudva et al., 1997). Even though cattle excrete *E. coli* O157 in their feces transiently for short periods of a few weeks, *E. coli* O157:H7 has been isolated from other environmental sources on farms for longer periods of up to several years (Hancock et al., 1997; Shere et al., 1998). The finding that *E. coli* O157:H7 is able to survive in the environment for many years suggests that it may be reintroduced into cattle

from different sources in the contaminated farm environment, including cattle water troughs.

Various strains of *E. coli*, including *E. coli* O157:H7, are able to grow in the environment with their survival times varying greatly, depending on the chemical composition or carbon content, temperature, pH, and microbial interactions (Lynn et al., 1998; Fenlon et al., 2000). Several factors, such as competition, predation and low nutrient concentrations, have been proposed which could considerably reduce the survival rates of bacteria in the aquatic environment. However, fecal bacteria have been shown to survive and grow in sediments (LaLiberte and Grimes, 1982; Davies et al., 1995). Some studies indicate that *E. coli* O157:H7 is very persistent and can survive for a long period of time in water and feces, especially at cold temperatures of 5°C compared to 20°C or 22°C (Wang and Doyle MP, 1998; Wang et al., 1996). In a study by LeJeune et al. (2001), it was shown that *E. coli* O157:H7 could survive in the sediments of chlorinated cattle water troughs for 245 days. In another study, *E. coli* O157:H7 survived for 77, >226, and 231 days in manure-amended autoclaved soil held at 5°C, 15°C, and 21°C, respectively. However, there was more rapid inactivation of *E. coli* O157:H7 in unautoclaved soil than in autoclaved soil at all three temperatures due to competition from other microorganisms (Jiang et al., 2002). The role of water troughs as reservoirs for *E. coli* O157:H7 and the frequency of waterborne transmission of these pathogens from water to cattle is not fully known. Hence, it is important to understand the survival characteristics of *E. coli* O157:H7 in these aquatic environments. The purpose of this study was to determine the survival and growth characteristics of different strains of *E.*

*coli* O157:H7 in cattle water sediments at different temperatures in the presence and absence of natural microbiota using experimental microcosms.

## MATERIALS AND METHODS

### Bacterial Strains

The five *E. coli* O157:H7 strains used in this study were: *E. coli* O157:H7 strain 301C (chicken isolate, *stx1*<sup>+</sup> *stx2*<sup>+</sup>), *E. coli* O157:H7 strain 204P (pork isolate, *stx1*<sup>+</sup> *stx2*<sup>+</sup>), *E. coli* O157:H7 strain F501 (*stx1*<sup>+</sup> *stx2*<sup>+</sup>), *E. coli* O157:H7 strain 43889 (human isolate, *stx2*<sup>+</sup>), and *E. coli* O157:H7 strain 43890 (human isolate, *stx1*<sup>+</sup>). All the five strains were obtained from the Food Safety/ Food Microbiology laboratory, Louisiana State University. The five strains were transformed using a green fluorescent protein (GFP) plasmid (Clonotech, Palo Alto, Calif.), according to the protocol described by Sambrook (2001) as follows:

Preparation of competent cells: A single bacterial colony from a TSA (Tryptic Soy Agar) plate incubated at 37°C for 16-20 h was transferred to a 100 ml LB (Luria-Bertani) broth in a 1-liter flask. The culture was incubated for 3 h at 37°C with agitation. Cells were transferred to sterile, ice-cold 50-ml tubes and cooled to 0°C for 10 min. Cells were then centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was decanted and the cell pellet was resuspended in 30 ml of ice-cold MgCl<sub>2</sub>-CaCl<sub>2</sub> solution (80 mM MgCl<sub>2</sub>, 20 mM CaCl<sub>2</sub>). The cells were pelleted again by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 2 ml of ice cold 0.1 M CaCl<sub>2</sub> for each 50 ml of original culture.

Transformation: 200 µl of the above cell suspension was transferred to a sterile tube and 10 µl of 50 ng DNA was added. The contents in the tube were mixed gently and stored on ice for 30 min. The tube was then placed in a 42°C water bath for 90 s. The tube

was then transferred to ice for 1-2 min. Eight hundred  $\mu\text{l}$  of LB broth was added to the tube and incubated for 45 min at 37°C. The transformed cells were then plated on LB medium containing 100  $\mu\text{g}/\text{ml}$  ampicillin and the plates were incubated at 37°C. The resulting ampicillin-resistant GFP-expressing *E. coli* O157:H7 strains emitted bright green fluorescence under a UV light at 365-nm wavelength.

### **Preparation of the Inoculum**

Two consecutive 24-h transfers of trypticase soy broth containing 100  $\mu\text{g}$  of ampicillin (TSB-A, 10  $\mu\text{l}$ ) per ml and incubated at 37°C were made for each strain immediately before the experiment. Each strain was then grown in 100 ml of trypticase soy broth containing 100  $\mu\text{g}$  of ampicillin (TSB-A) per ml for 18 h at 37°C without agitation. The bacteria were pelleted by centrifugation (5,000  $\times g$ , 10 min), washed three times in 0.1 M phosphate-buffered saline, pH 7.2 (PBS), and resuspended in PBS. Cells were diluted with PBS to an optical density at 630 nm of 0.7 and verified by enumeration on TSA-A plates.

### **Sample Preparation**

Sediment and algal biofilm samples were collected from cattle water troughs at one of the cattle farms in Louisiana State University research stations. To simulate conditions in cattle water troughs, biofilm samples were combined with an equal weight of sediments freshly collected from cattle water troughs. This mixture was equally divided into 198 g aliquots, distributed into sterile containers (microcosms) and assigned into two treatment groups either with or without natural microbiota to determine the effects of microbial competition on the growth and survival of different strains of *E. coli* O157:H7. The treatment group with natural microbiota consisted of aliquots of

sediment/biofilm samples that were left untreated and the other treatment group without natural microbiota consisted of aliquots of sediment/biofilm samples that were sterilized by autoclaving before being added to each microcosm. One hundred milliliters of sterilized water collected from water troughs was then added to each microcosm. Before inoculation, a 10-g sample of sample was tested for the presence of *E. coli* O157:H7 by enrichment and nonenrichment methods as follows:

Enrichment method: A 10 g aliquot of each sample was enriched with an equal volume of a 2x concentration of TSB. Enrichments were incubated overnight at 37°C. Dilutions of overnight enrichments were made and spread plated onto Sorbitol MacConkey agar plates containing 2.5 µg of potassium tellurite/ml and 50 ng of cefixime/ml (SMAC- CT). Following incubation, colonies exhibiting morphology typical of presumptive sorbitol negative *E. coli* O157:H7 (gray or pale with a darker center) were tested for indole (addition of 0.2-0.3 ml Kovacs' reagent to 5 ml of 24 h tryptophane broth inoculated with the test bacteria). All SMAC colonies that were found to be sorbitol negative and indole positive (dark pink color of tryptophane broth) were checked for agglutination with O157 and flagellar H7 antiserum according to the manufacturer's recommendations (Remel Inc., Lenexa, USA). Morphologically typical, indole-positive, non-sorbitol-fermenting colonies which were positive for O157 latex agglutination were considered presumptive *E. coli* O157:H7 based on culture and latex agglutination. The same method was followed for detection by nonenrichment method except that the samples were analyzed without overnight incubation in TSB at 37°C.

### **Inoculation, Incubation and Sampling**

An inoculum (2 ml) of each of the five strains of *E. coli* O157:H7 ( $\sim 10^9$  CFU/ml) was added to each separate aliquot of 198 g of the sediment/biofilm sample and mixed thoroughly in sterile stomacher bags for 4 min in a stomacher to obtain a bacterial concentration of  $\sim 10^7$  CFU/g. The inoculum was added to samples of both treatment groups (microcosms with and without natural microbiota). Each sample was inoculated with only one strain of *E. coli* O157:H7. The microcosms (with and without natural microbiota) were loosely covered with lids and incubated at 5°C, 15°C, 25°C and 37°C after inoculation. At all temperatures, duplicate samples were incubated and the samples were cultured to determine the presence of *E. coli* O157:H7 periodically for two months. *E. coli* O157:H7 counts were determined at 0, 1, 3, 5, and 7 days after inoculation and then once every week for two months. When the pathogen could no longer be isolated by direct detection methods, samples were analyzed by selective enrichment. The initial (zero-time postinoculation) *E. coli* O157:H7 concentration was determined for each microcosm prior to incubation at various temperatures.

### **Detection of *E. coli* O157:H7 in the Microcosms**

At each sampling time, 10 ml of sterile 0.1 M phosphate-buffered saline, pH 7.2 (PBS), solution was added to a 1-g aliquot of each sediment/biofilm sample, and the diluted samples were mixed thoroughly by vortexing. Additional 10-fold serial dilutions of the homogenized sample were made in 0.1 M phosphate-buffered saline, pH 7.2 (PBS) solution and *E. coli* O157:H7 counts were determined by plating 0.1 ml portions of each dilution on TSA-A plates, which were incubated at 37°C for 24 h. The GFP-labeled *E. coli* O157:H7 colonies were counted under a UV light. When *E. coli* O157:H7 colonies

were not detectable by direct plating, cells were detected by selective enrichment as follows. A 1-g sample was added to 99 ml of TSB-A and incubated at 37°C for 24 h. Dilutions of the enrichment cultures were then surface plated on TSA-A plates. Colonies that were green and fluoresced under UV light were counted as *E. coli* O157:H7.

Randomly selected green fluorescent colonies were confirmed to be *E. coli* O157:H7 by latex agglutination test. All tests were performed in duplicate. Confirmed *E. coli* O157:H7 colonies obtained from the longest survival period at each incubation temperature were assayed for verotoxin production. The presence of toxin genes in each of the five strains of *E. coli* O157:H7 was also monitored every two weeks for a period of two months.

#### **Detection of *stx1* and *stx2* Genes**

PCR detection of *stx1* and *stx2* genes were performed by a GeneAmp PCR thermocycler (Model 2400, Perkin-Elmer, USA). Oligonucleotide primers were purchased from Integrated DNA Technologies, Inc., Coralville, Iowa. The oligonucleotide sequence of primers used were as follows: *stx1* Forward: CAGTTAATGTCGTGGCGAAGG; *stx1* Reverse: CACCAGACAATGTAACCGCTG  
*stx2* Forward: ATCCTATTCCCGGGAGTTTACG; *stx2* Reverse: GCGTCATCGTATACACAGGAGC (Osek, 2003). Crude template DNA for each isolate was prepared by adding 25 µl of an overnight culture to 500 µl of sterile distilled water and boiling at 95°C for 5 min. The PCR assays was performed in 50 µl reaction volume containing 25 µl Taq PCR Master Mix (premixed solution containing 5 units/µl *Taq* DNA Polymerase, PCR buffer containing both KCl and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub> and 200 µM each dNTP; Qiagen Inc., USA), 3 µl (0.5 µM) of primer, 14 µl of Millipore

water, and 5 µl of template DNA. The PCR thermocycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 1 min (Osek, 2003). The final extension step was at 53°C for 5 min. The amplified PCR products were separated on 1.5% agarose gels in TBE (Tris-Borate-EDTA) buffer, followed by staining with SYBR® Gold nucleic acid gel stain (Molecular Probes, Invitrogen, USA) and photographed under UV illumination.

### **Vero Cell Cytotoxic Assay**

A continuous African green monkey kidney (Vero) cell line was maintained by weekly trypsinization of confluent monolayers grown in Eagles's Minimum Essential Medium (EMEM; ATCC, USA) containing 10% fetal calf serum. The Vero cell assay was performed on aliquots of filter-sterilized mitomycin C treated enrichment broth after centrifugation. Fresh monolayers of Vero cells (ATCC, USA) were prepared by suspending 200 µl of  $10^5$  cells/ml in 96-well polystyrene cell culture plates. The cells were allowed to grow for 2 days, at which time fresh growth medium (200 µl per well) was added. Culture filtrate (50 µl) of individual isolates was added to duplicate wells with confluent monolayers. Culture filtrate was obtained by growing each isolate in TSB at 37°C for 24 h, removing the cells by centrifugation (7000 x g, 10 min), and filtering the supernatant fluid through a 0.2 µm-pore size membrane filter. Plates were sealed, incubated in a carbon dioxide enriched atmosphere (5%, w/v) at 37°C, and then examined under an inverted microscope after 48 h. In the presence of shiga toxins, the monolayer is disrupted and cells become detached. Isolates were considered to be positive for toxin production when 50% of the Vero cells were dead and detached.

## Statistical Analysis

The experiment was performed in two replicates with two samples being evaluated for each replicate. Data were analyzed by statistical comparisons of all pairs using one-way analysis of the variance (ANOVA; JUMPIIn version 4.0.3, SAS Institute Inc., Cary, N.C., U.S.A.) to determine significant differences ( $P \leq 0.05$ ) in survival of different strains of *E. coli* O157:H7 at different temperatures.

## RESULTS

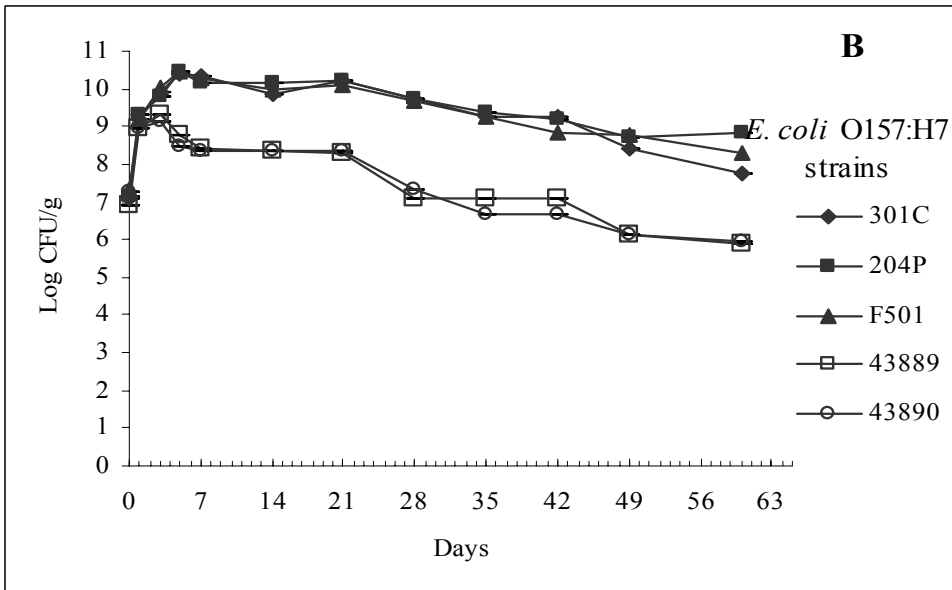
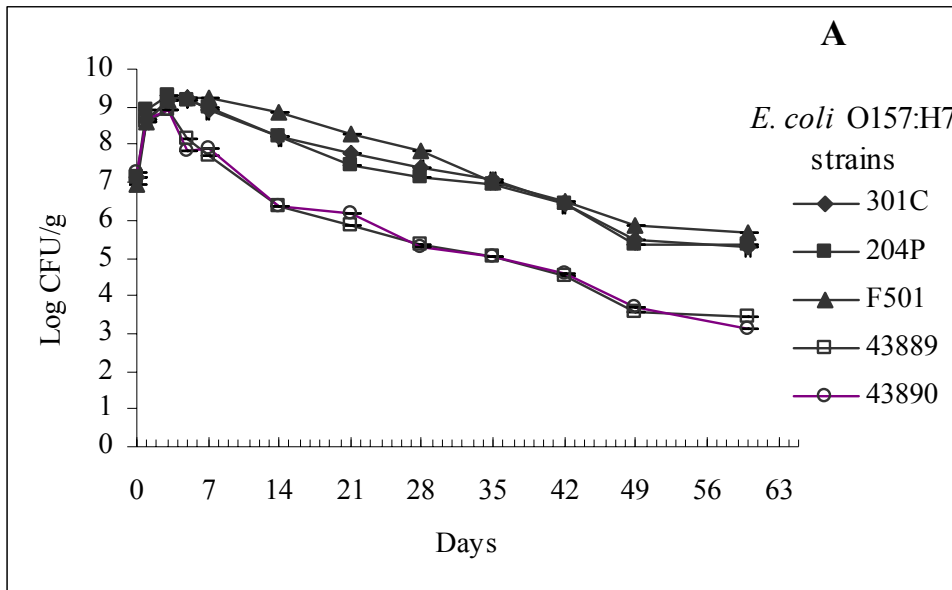
The stability of the GFP in the five *E. coli* O157:H7 strains was determined by plating each isolate on TSA containing 100 µg of ampicillin per ml (TSA-A) for several generations before the start of the experiment. Even after repeated streaking on TSA-A plates, all the five GFP-labeled *E. coli* O157:H7 strains still retained the GFP label. GFP-labeled *E. coli* O157:H7 colonies on TSA-A plates, emitted bright green fluorescence under UV light and were very easily distinguishable from the natural flora present in the sediments. The *gfp* gene has been used to label *E. coli* O157:H7 enabling them to be distinguished from other background microflora (Ehrmann et al., 2001; Jiang et al., 2002). Ampicillin-resistant organisms were recovered and the resistance was maintained by all bacterial strains throughout the test period. Recovery of the *E. coli* O157:H7 on TSA without ampicillin was not significantly different ( $P \leq 0.05$ ) from that on TSA with ampicillin indicating adequate recovery of any stressed test bacteria.

Experimental microcosms simulating cattle water troughs were used to evaluate survival patterns of different strains of *E. coli* O157:H7 in the presence and absence of natural microflora at four temperatures (5, 15, 25, and 37°C). The five strains of *E. coli* O157:H7 used in this study originated from different sources. Strains 301C and 204P

were chicken and pork isolates respectively, strain F501 was a CDC isolate and strains 43889 and 43890 were human isolates. The average initial concentration of *E. coli* O157:H7 in all the samples after inoculation was  $1.5 \times 10^7$  CFU/g. Inoculated microcosms consisting of eight different treatment conditions for each strain of bacteria were incubated and the survival of *E. coli* O157:H7 was monitored for 60 days. No *E. coli* O157:H7 was detected in the sediment or biofilm samples before inoculation as determined by culturing.

### **Survival of *E. coli* O157:H7 Strains at 5°C**

The populations of all five strains of *E. coli* O157:H7 were similar only for the first three days of incubation for both treatments--with and without natural microbiota (Fig. 4.1). Continued incubation at 5°C for the rest of the 2-month period produced almost similar survival patterns for three strains of *E. coli* O157:H7 (301C, 204P, F501) and significantly lower survival rates for strains 43889 and 43890. There were no significant ( $P > 0.05$ ) differences between the survival patterns of strains 43889 and 43890. However, there were significant differences ( $P \leq 0.05$ ) in survival patterns of each of the strains of *E. coli* O157:H7 growing in treatments with and without the presence of competing microorganisms. All five *E. coli* O157:H7 were isolated from both the treatment conditions (with and without natural microbiota) at all sampling times throughout the 2-month period. The average population levels of *E. coli* O157:H7 strains 301C, 204P, F501 in the treatments with natural microflora had increased to 9.2 log CFU/g after 5 days and then decreased thereafter to an average of 5.32 log CFU/g at the end of 60 days. The average levels of strains 43889 and 43890 in the presence of competing microorganisms increased to 7.9 log CFU/g and decreased to an average of

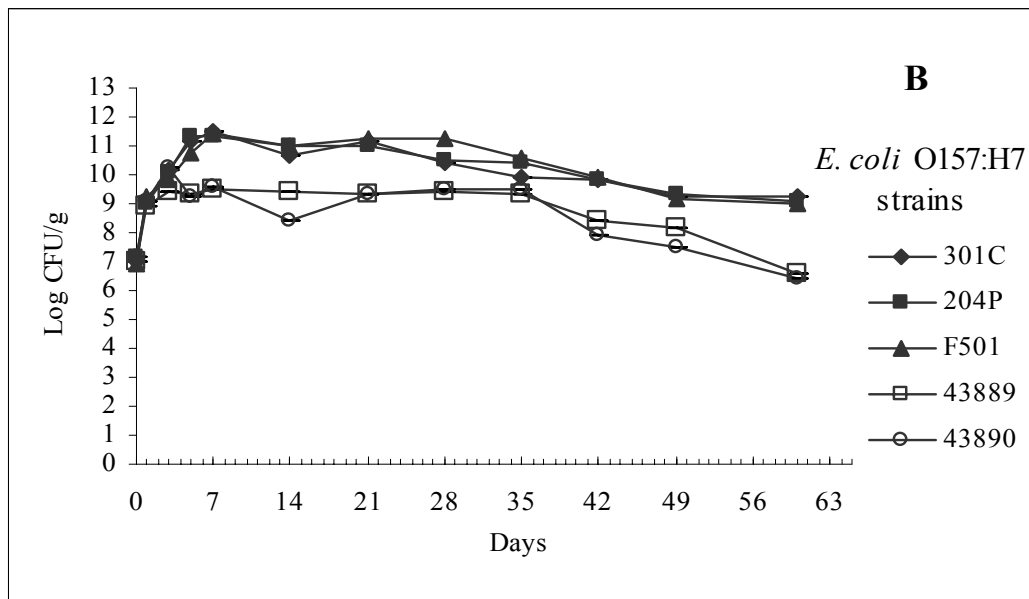
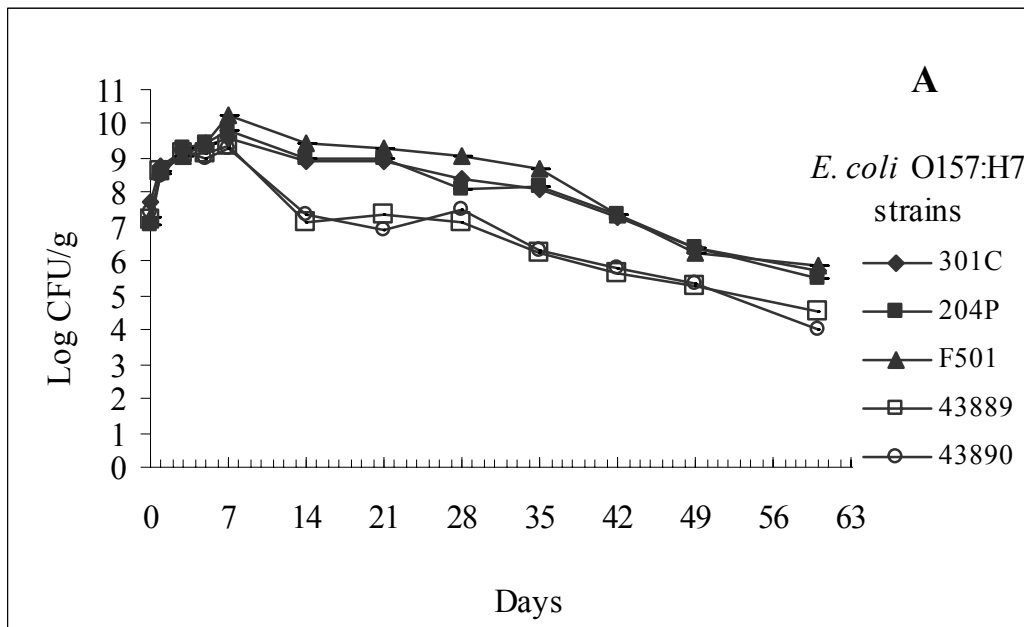


**Fig. 4.1. Survival of different strains of *E. coli* O157:H7 in sediments at 5°C.**  
 (A) Survival in the presence of natural microflora (B) Survival in the absence of natural microflora. Each point represents the average for duplicate experiments.

3.2 log CFU/g after 60 days of incubation. In the absence of competing microorganisms, however, the average levels of strains 301C, 204P, F501 increased to a maximum of 10.4 log CFU/g after 5 days and reduced to an average of 8.2 log CFU/g after 60 days, while strain 43889 and 43890 levels increased to an average of 8.5 log CFU/g and 5.9 log CFU/g after 5 and 60 days respectively.

### **Survival of *E. coli* O157:H7 Strains at 15°C**

Similar population levels of the five strains were isolated from treatments with natural microbiota at 15°C for at least one week postinoculation and for 3 days for treatments without natural microbiota (Fig. 4.2). Thereafter, there was a significant difference ( $P \leq 0.05$ ) in the patterns of recovery for the five strains of *E. coli* O157:H7 incubated both with and without natural microbiota. Incubation for the rest of the 2-month period showed similar patterns of survival for three strains of *E. coli* O157:H7 (301C, 204P, F501) and significantly ( $P \leq 0.05$ ) lower survival rates for strains 43889 and 43890 with no significant ( $P > 0.05$ ) differences between strains 43889 and 43890. The average population levels of strains 301C, 204P, F501 in the presence of natural microbiota reached a maximum of 9.5 log CFU/g after 7 days and started to decrease and reached an average level of 5.7 log CFU/g after 60 days, whereas strains 43889 and 43890 grew to a maximum level of 9.3 log CFU/g after 7 days and decreased to 4.3 log CFU/g after 60 days. In the absence of competing microorganisms, the average population levels of strains 301C, 204P, F501 reached a maximum of 11.4 log CFU/g after 7 days and decreased to an average count of 9.0 log CFU/g after 60 days, whereas strains 43889 and 43890 grew to a maximum level of 9.5 log CFU/g after 7 days and decreased to 6.4 log CFU/g after 60 days.



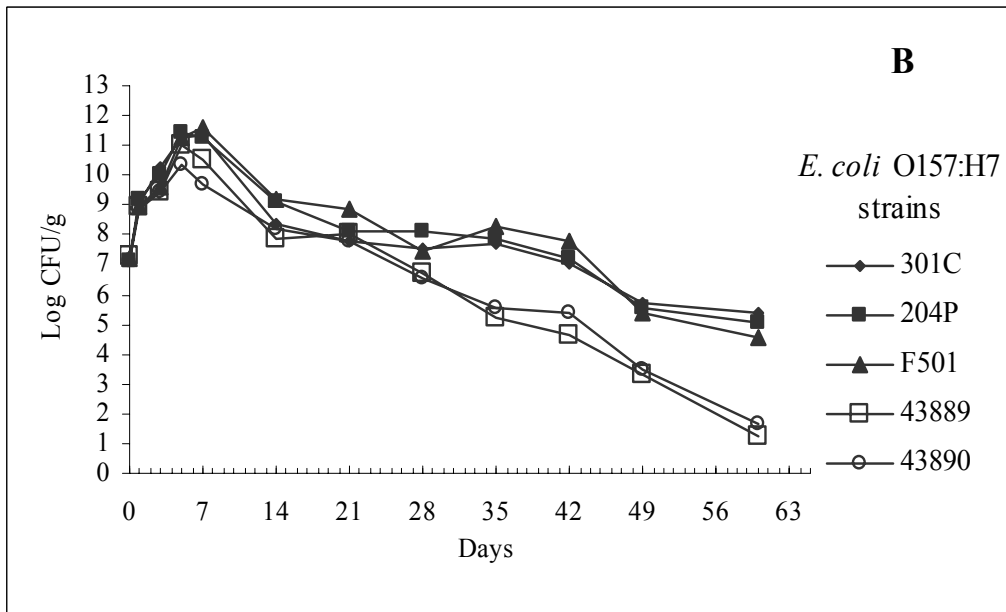
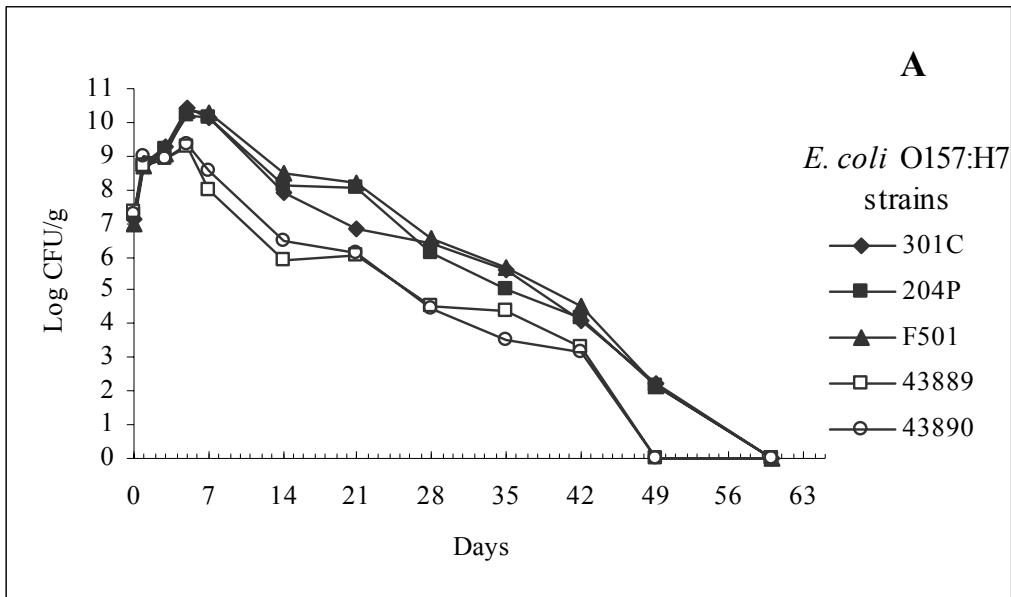
**Fig. 4.2. Survival of different strains of *E. coli* O157:H7 in sediments at 15°C.** (A) Survival in the presence of natural microflora (B) Survival in the absence of natural microflora. Each point represents the average for duplicate experiments.

### **Survival of *E. coli* O157:H7 Strains at 25°C**

At 25°C, there was no significant difference between the population levels of the five strains for 3 days in treatments with natural microbiota and for 21 days for treatments without natural microbiota (Fig. 4.3). Subsequently, there were significant differences ( $P \leq 0.05$ ) in the patterns of recovery of the five strains of *E. coli* O157:H7 incubated with and without natural microbiota. Incubation for the rest of the 2-month period showed no significant difference in populations levels between the three strains of *E. coli* O157:H7 (301C, 204P, F501) whereas 43889 and 43890 strain populations were significantly ( $P \leq 0.05$ ) lower from the other three strains. In the presence of competing microbes, the average population of strains 301C, 204P, F501 reached a maximum of 10.2 log CFU/g after 5 days and decreased to non-detectable levels after 60 days, while strains 43889 and 43890 grew to a maximum level of 9.2 log CFU/g after 5 days and decreased to non-detectable levels after 49 days of incubation by direct culture method. All five strains could, however, be recovered by the selective-enrichment culture method for the rest of the study period. In the absence of competing microorganisms, the average concentrations of strains 301C, 204P, F501 reached a maximum of 11.5 log CFU/g after 7 days and decreased to an average concentration of 5.1 log CFU/g after 60 days, whereas strains 43889 and 43890 population increased to a maximum concentration of 9.5 log CFU/g after 5 days and decreased to 1.5 log CFU/g after 60 days.

### **Survival of *E. coli* O157:H7 Strains at 37°C**

At 37°C, the *E. coli* O157:H7 populations were similar for all the five strains for treatments with and without natural microbiota through most of the incubation period. During the first 3 days, *E. coli* O157:H7 increased in numbers by 100 to 1000-fold (Fig.



**Fig. 4.3. Survival of different strains of *E. coli* O157:H7 in sediments at 25°C.**  
 (A) Survival in the presence of natural microflora (B) Survival in the absence of natural microflora. Each point represents the average for duplicate experiments.

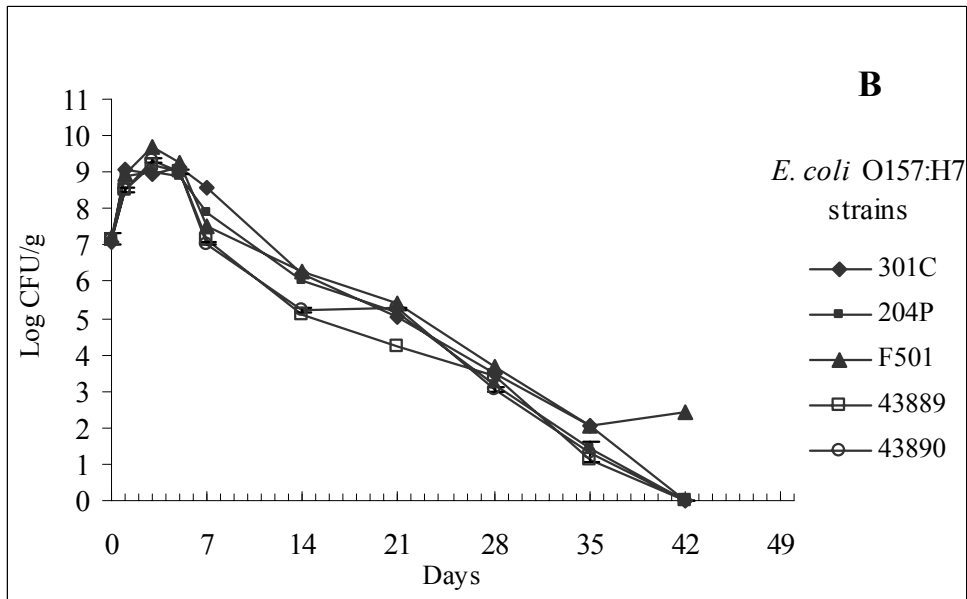
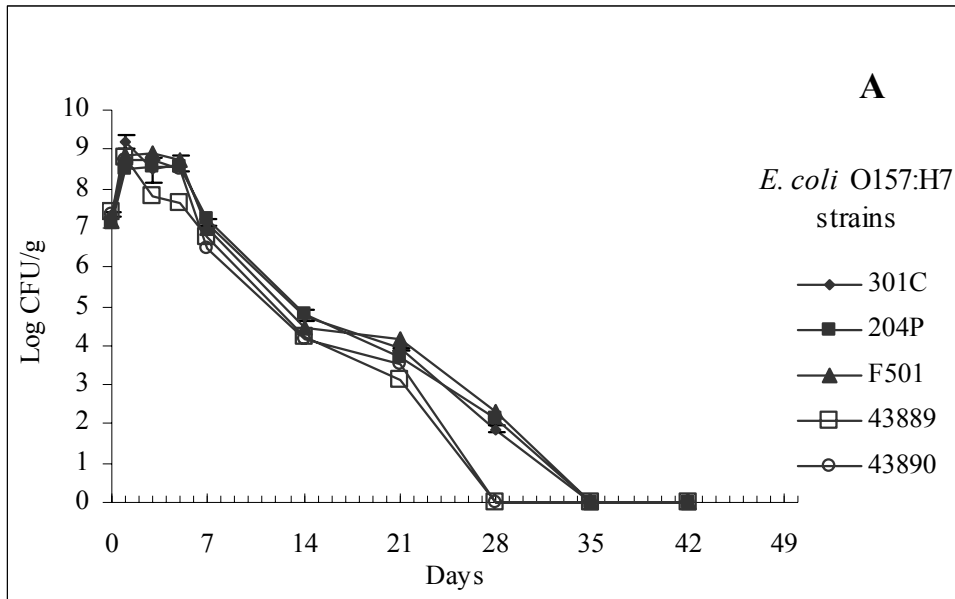
4.4). Consequently, *E. coli* O157:H7 levels declined differently depending upon treatment with highest populations in treatments without natural microbiota through the duration of the experiment. Strains 43889 and 43890 were non-detectable by direct plating after 28 days, whereas strains 301C, 204P, F501 were non-detectable after 35 days in the presence of competing microorganisms. Strains 301C, 204P, F501 could be detected by enrichment for 49 days, whereas strains 43889 and 43890 could be recovered by enrichment only for 42 days. In the absence of competing microorganisms, all strains could be detected by direct plating for 35 days and could be isolated by an enrichment procedure after 49 days postinoculation. Strain F501 was the only surviving strain detected by direct plating after 42 days of incubation and could be detected by enrichment after 60 days.

#### **Toxin Production and Toxin genes**

All of the strains from each incubation temperature retained the toxin genes and the ability to produce the shiga toxins as determined by PCR and Vero cell assay respectively until the end of the incubation period.

### **DISCUSSION**

Laboratory experiments were set up to evaluate survival of different strains of *E. coli* O157:H7 in the sediments of cattle water troughs at four different temperatures. During the first 3 days, populations of the all the five *E. coli* O157:H7 strains increased at all temperatures indicating growth of the pathogen in sediments at these temperatures. Thereafter, *E. coli* O157:H7 populations decreased more rapidly at higher temperatures (25°C and 37°C) than at lower temperatures (5°C and 15°C). The pathogens were able to survive even in the presence of competing microorganisms at lower temperatures till the



**Fig. 4.4. Survival of different strains of *E. coli* O157:H7 in sediments at 37°C.**  
 (A) Survival in the presence of natural microflora (B) Survival in the absence of natural microflora. Each point represents the average for duplicate experiments.

end of the study period. All the strains incubated in treatments at 15°C were recovered at higher levels than in treatments at 5, 25 or 37°C. These findings are similar to the findings of Wang et al. (1996) who found that the survival rates of *E. coli* O157:H7 in bovine feces varied depending on temperature and also that *E. coli* O157:H7 survived for the longest time (70 days) at 5°C than at higher temperatures. In a similar study, Kudva et al. (1998) found that *E. coli* O157:H7 survived better in farm effluents at temperatures below 23°C (-20, 4, or 10°C) than in effluents incubated at higher temperatures (37, 45, or 70°C).

The populations of all five strains were higher in sediments without natural microflora than in sediments with natural microflora. *E. coli* O157:H7 levels declined more rapidly at higher temperatures than at lower temperatures in treatments with natural microflora probably because of the slow or lack of growth of competing microorganisms at low temperatures.

There was no significant difference between the average responses of the three strains (301C, 204P, F501) that were originally isolated from chicken, pork and the CDC respectively at 5, 15, and 25°C temperatures either in the presence or absence of competing microorganisms. These three isolates, on average, survived at higher levels than the human isolates (43889 and 43890) at 5, 15, and 25°C. There was no significant difference in survival of the five strains at 37°C; however, the strains 301C, 204P, F501 survived longer than the human isolates at 25°C and 37°C. Similar differences in survival between environmental and human isolates were observed in a study by Avery and Buncic (2003) who hypothesized that human isolates were significantly more sensitive to environmental stresses such as drying than the environmental isolates

probably because the human isolates were more adapted to the warm, moist environment of the human gastrointestinal tract and less able to survive in the colder, dryer environment of the farms. Although this study provided further evidence of survival differences between the human isolates and the non-human isolates, it was not possible to confirm the above hypothesis based on our results.

Survival and proliferation of *E. coli* O157:H7 under different environmental stresses such as low pH (Arnold and Kaspar, 1995), low nutrients (Wang and Doyle, 1998; Warburton et al., 1998), low  $a_w$  (Wang et al., 1996) and chlorination (LeJeune et al., 2001) has been well documented. *E. coli* O157:H7 strain differences in growth and survival patterns in response to environmental stresses such as drying and acidic conditions were also found by Wang et al. (1996) and Arnold and Kaspar (1995) respectively.

The ability of *E. coli* O157:H7 to persist in the sediments of cattle water troughs as shown in this research is supported by recent studies that have shown a higher prevalence of *E. coli* O157:H7 in cattle water troughs (LeJeune et al. 2004; Van Donkersgoed et al., 2001) compared to the prevalence in other farm environments such as cattle feces and feed bunks. The prevalence of *E. coli* O157:H7 in water troughs of two feedlots was found to be as high as 22% in the summer (Van Donkersgoed et al., 2001) compared to 0.8% in cattle feces and 1.7% in feed bunks. In a study of a commercial beef feedlot in the summer months, LeJeune et al. (2004), found a prevalence rate of 26% and 17% in water troughs supplied with unchlorinated water and chlorinated water respectively and a prevalence of 13.3% in cattle feces. In both these studies (Van Donkersgoed et al., 2001; LeJeune et al., 2004) feces, water troughs, and feedbunks were

shown to have some common subtypes of *E. coli* O157:H7, suggesting transmission of *E. coli* O157:H7 among these sites. In another study by LeJeune et al. (2001), it was found that bacterial contamination was higher in water troughs that were closest to the feedbunk and also that *E. coli* O157:H7 was isolated more frequently in the less recently cleaned water troughs. These studies provide evidence that cattle water troughs provide environments conducive for growth and survival of *E. coli* O157:H7.

Some studies have reported instability and loss of Shiga toxin genes in laboratory-propagated cultures (Murase et al., 1999; Iguchi et al., 2002; Shaikh and Tarr, 2003). Results from our study showed that none of the strains lost the toxin genes or the ability to produce toxins throughout the study period under all treatment conditions. Similar results were obtained by Wang et al. (1996) who found that none of the five strains incubated in cattle feces for more than 10 weeks at different temperatures lost the ability to produce toxins. In a study by Kudva et al. (1998), it was also shown that *E. coli* O157:H7 can persist in nonaerated manure pile for more than 12 months and that the DNA fingerprints (PFGE genomic DNA profiles) of *E. coli* O157:H7 isolated after one month were identical to those isolated after 12 months. LeJeune et al. (2001) also found identical genetic profiles for the *E. coli* O157:H7 strain used in their microcosm studies even after 6 months.

Bacteria in aquatic environments are more likely to proliferate as the water temperature increases above 15°C (LeChevallier et al., 1996). It is, therefore, hypothesized that the increase in infection of cattle with *E. coli* O157:H7 during summer months may be partly due to increase in concentrations of this pathogen in contaminated water troughs (Van Donkersgoed et al., 2001). Although several studies (Hancock et al.,

1997; Dargatz et al., 1997; Van Donkersgoed et al., 2001) have reported a high prevalence of *E. coli* O157:H7 in cattle water troughs in the summer months compared to the winter months, results from our study show that *E. coli* O157:H7 can survive and grow better at lower temperatures (5°C and 15°C) than at higher temperatures (25°C and 37°C). This study suggests that factors such as temperature, competing microflora, and strain differences play an important role in the survival of *E. coli* O157:H7 in the sediments of water troughs. Several studies have shown an association between the presence of *E. coli* O157:H7 in cattle water troughs and the infection status of cattle drinking from these troughs (Shere et al., 2002; Shere et al. 1998; Faith et al., 1996). Therefore, improper sanitary conditions in cattle water troughs contaminated with *E. coli* O157:H7 may serve as a reservoir for this pathogen for long periods of time.

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## CHAPTER 5

### EFFECTS OF CETYLPYRIDINIUM CHLORIDE AGAINST *ESCHERICHIA COLI* O157:H7 BIOFILMS ON STAINLESS STEEL

## INTRODUCTION

Bacteria have the ability to attach to a variety of surfaces and form biofilms that persist in the food environments and contaminate food during processing. Animal and plant food used for human consumption carry a wide range of microorganisms at the time of slaughter or harvest that differ in numbers and types depending on the type of food, the geographical origin of the food, and the agricultural practices used in production. During processing, food products may be contaminated either directly with organisms from the raw material or indirectly by indigenous bacteria that persist in the food processing facilities. Bacterial biofilms on food or the food contact surfaces leads to serious hygienic problems and economic losses due to food spoilage.

According to CDC (The Center for Disease Control and Prevention) estimates, the annual cost of illness due to *Escherichia coli* O157:H7 infections acquired from food or other sources is \$405 million (Frenzen et al., 2005). Due to the high cost of illness due to *E. coli* O157: H7 infections, the federal government has implemented mandatory hazard analysis and critical control point (HACCP) programs and improved pre- and post-harvest processes to lower the contamination of meat products with pathogens such as *E. coli* O157:H7 in meat and poultry plants and the juice industry (Frenzen, 2005).

Most outbreaks of *E. coli* O157:H7 have been the associated with foods of bovine origin. In cases involving non-bovine foods, cross contamination by beef or contamination with bovine feces during processing has often been suspected. It is believed that *E. coli* O157:H7 contamination of foods occurs from the intestinal tract of healthy cattle during slaughter and processing (Wells et al., 1991; Wang et al., 1996). Cross-contamination in abattoirs (Bouvet et al., 2001; Warriner et al., 2002) and other

food processing plants (Beuchat and Ryu, 1997; Warriner et al., 2002) have been reported to be responsible for several outbreaks of *E. coli* O157:H7.

*E. coli* O157:H7 is known to produce extracellular polymeric substances (EPS) and form biofilms on food processing surfaces (Junkins and Doyle, 1992; Dewanti and Wong, 1995; Ryu et al., 2004). Some biotic and abiotic factors that influence biofilm formation include, the growth phase of cells, the type and properties of the inert surface, the presence of organic nutrients, environmental pH, and temperature 2004 (Kumar and Anand, 1998). In a study by Hassan and Frank (2003), *E. coli* O157:H7 biofilm formation has been shown to be influenced by hydrophobicity, surface charge and capsule production. Microorganisms are known to be more resistant to removal from foods and food contact surfaces in processing plants and to inactivation by sanitizers when contained in a biofilm than when dispersed in a liquid medium (Kumar and Anand, 1998; Carpentier and Cerf, 1992). *E. coli* O157:H7 growing in biofilms in the food processing environments have been reported to develop resistance to various environmental stresses such as sanitizers, low temperatures and pH (Stopforth et al., 2003; Samelis et al., 2005).

Quaternary ammonium compounds (QACs), a group of antiseptics and disinfectants, are membrane active agents and are known to lower cellular surface tension, disrupt the bacterial cell membrane, and cause loss of selective permeability of the bacterial cell membrane. QACs are frequently being used as disinfectants in food industries to prevent the spread of microorganisms (Merianos, 1991). Cetylpyridinium chloride (CPC) is a water-soluble, colorless QAC that is currently used in commercial mouthwashes at a maximum permitted concentration of 0.1% by the U.S. Food and Drug Administration. Currently, it is only approved for use in poultry processing (FDA, 1998).

Various studies have been conducted in which CPC has been successful in reducing bacterial counts of different pathogens on food surfaces (Kim and Slavik, 1996; Breen et al., 1997; Xiong et al., 1998; Bosilevac et al., 2004). Kim and Slavik (1996) reported reduction of *Salmonella* Typhimurium populations on chicken skins by 2.5 log<sub>10</sub> using 30s spray of 0.1% CPC. Breen et al. (1997), showed that 0.4% CPC reduced *Salmonella typhimurium* counts on poultry surfaces by 4.9 log<sub>10</sub> using 0.4% CPC in 3 min. Other studies have shown that CPC is effective in reducing or inhibiting gingivitis and plaque formation at concentrations of 0.05 to 0.5% (Kozlovsky et al., 1994; Frost and Harris, 1994).

The objective of this study was to determine the effects of temperature and culture conditions on biofilm formation by *E. coli* O157:H7 on stainless steel and to investigate the antimicrobial effects of CPC on these biofilms.

## **MATERIALS AND METHODS**

### **Bacterial Strains and Culture Conditions**

A five-strain mixture of *E. coli* O157:H7 was used in this study. The five strains included, *E. coli* O157:H7 strain 301C (chicken isolate, *stx1*<sup>+</sup> *stx2*<sup>+</sup>), *E. coli* O157:H7 strain 204P (pork isolate, *stx1*<sup>+</sup> *stx2*<sup>+</sup>), *E. coli* O157:H7 strain F501 (*stx1*<sup>+</sup> *stx2*<sup>+</sup>), *E. coli* O157:H7 strain 43889 (human isolate, *stx2*<sup>+</sup>), and *E. coli* O157:H7 strain 43890 (human isolate, *stx1*<sup>+</sup>). All the five strains were obtained from the Food Safety/ Food Microbiology laboratory, Louisiana State University. Stock cultures were maintained at -70°C and working cultures were maintained on trypticase soy agar (TSA) at 4°C. Two consecutive 24-h transfers of trypticase soy broth (TSB, 10 µl) incubated at 37°C were made immediately before the experiment. Each strain was then grown separately in 250

ml of TSB for 24 h at 37°C with slight agitation. The bacteria were pelleted by centrifugation (5000 x g for 10 min), washed three times in 0.1 M phosphate-buffered saline (PBS, pH 7.4), and resuspended in PBS. Cells were adjusted with PBS to an optical density at 640 nm of 0.5 (approximately 10<sup>8</sup> CFU/ml). The five strains were then combined in equal concentrations. The populations of each individual strain and the five-strain mixture were verified by enumeration on TSA.

### **Media Used**

Tryptic soy broth was used to culture *E. coli* O157:H7 for use in the biofilm study and TSA was used to enumerate the bacteria. Three types of media were used to determine the effects of nutrients on biofilm formation by *E. coli* O157:H7 on stainless steel surfaces and the antimicrobial effects of cetylpyridinium chloride (CPC) on these biofilms—TSB, 10% TSB, and minimal salts broth (MSB, pH 7; Difco, USA).

### **Preparation of Sanitizer and Neutralizer Solutions**

Cetylpyridinium chloride (CPC) solutions (0.01%, 0.05%, 0.5%, 1.0%; Safe Foods Corp., North Little Rock, AR) were made with sterile distilled water according to the manufacturer's instructions. Dey-Engley (D/E, pH 7.4; Difco, USA) neutralizing broth was used to neutralize the CPC after treating biofilms on the stainless steel chips.

### **Preparation of Stainless Steel Chips**

Stainless steel chips (type 304 with #4 finish, 3 by 1 cm) were washed in an alkaline detergent (Alconox, Alconox, Inc., New York, N.Y.) and then rinsed thoroughly with distilled water. The chips were then dry sterilized in an autoclave at 121°C for 15 min before use.

## **Development of Biofilm on the Surface of Stainless Steel and CPC Treatment**

To attach cells in the suspension onto the stainless steel chips, 10 ml of five-strain cell suspension was placed in a 15-ml tube containing a sterile chip and incubated at 22°C for 4h. After incubation, each chip was rinsed in sterile distilled water to remove loosely attached cells on the surface of the chips. The chips were then transferred to 15 ml tubes containing 10 ml of TSB, 10 ml of 10% TSB, or 10 ml of MSB and incubated at 12°C and 22°C. The chips containing attached biofilms were then removed after 2, 10, and 21 days and rinsed with sterile distilled water individually to remove any reversibly attached bacteria. Chips were then immersed in 30 ml CPC solutions (only one chip for each 30 ml solution) at each CPC concentration (0.01%, 0.05%, 0.5%, 1.0%) for 2, 5, and 10 min. After the CPC treatment, the chips were transferred to tubes containing 10 ml of sterile D/E neutralizing broth and 3 g of glass beads (2 mm diameter; Fisher Scientific, USA) and vortexed at maximum speed for 2 min to remove attached cells from the surface of the chips. The cells suspended in the D/E broth were enumerated by plating serial dilutions on TSA and incubated at 37°C for 24 h to determine total cell counts per chip (log CFU/chip). To evaluate biofilm formation before the sanitizer treatment, the chips with adherent cells were placed directly into the D/E broth without sanitizer treatment, vortexed along with sterile glass beads and enumerated by plating serial dilutions on TSA.

## **Statistical Analysis**

The experiment was replicated twice with three samples being evaluated for each replicate. Data were analyzed by statistical comparisons of all pairs using one-way analysis of the variance (ANOVA; JUMPIIn version 4.0.3, SAS Institute Inc., Cary, N.C.,

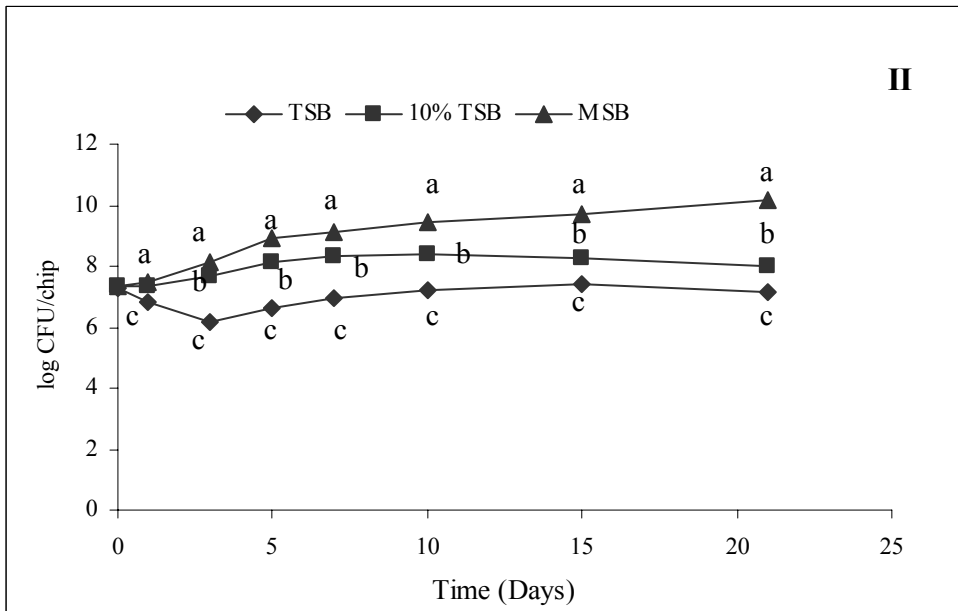
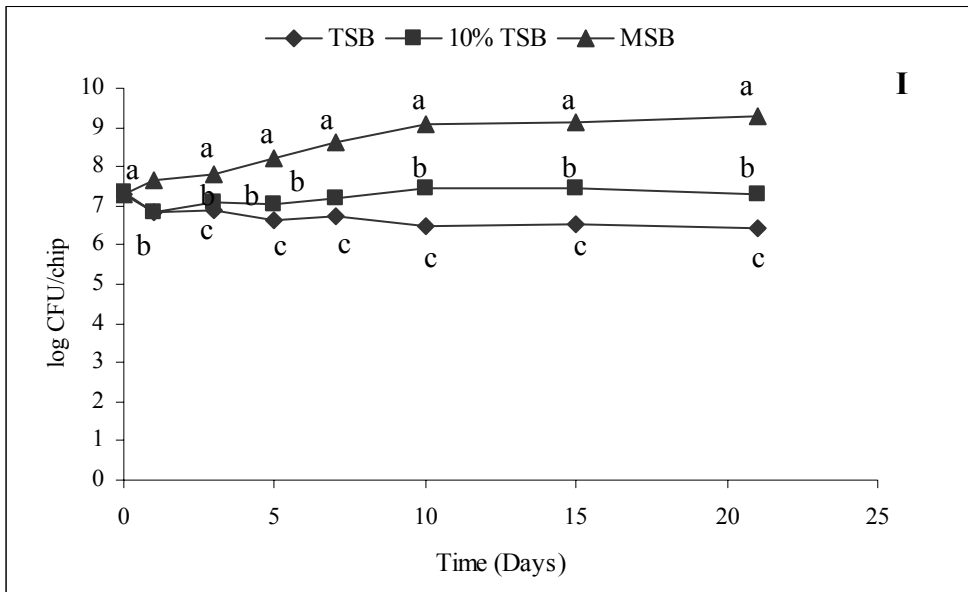
U.S.A.) to determine significant differences ( $P \leq 0.05$ ) in total cell counts as affected by incubation in TSB, 10% TSB and MSB for 21 days. For biofilms treated with CPC, the total cell counts after treatment for a given time and concentration of CPC were compared for significant differences ( $P \leq 0.05$ ).

## RESULTS

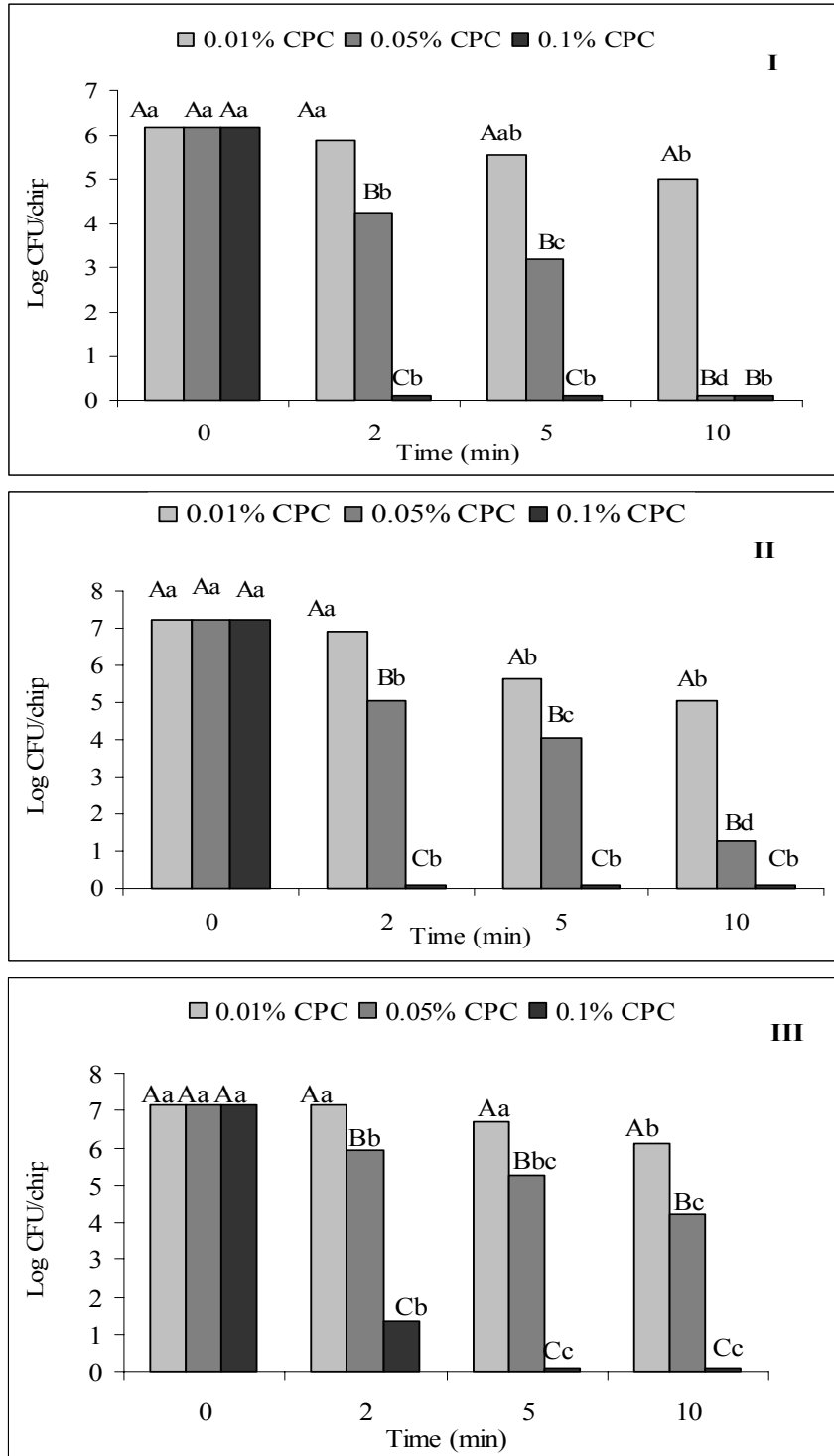
### **Biofilm Formation by *E. coli* O157:H7 on the Surface of Stainless Steel Chips Placed in Different Culture Media**

The total cell count on the chips at the end of the 4-h attachment period was 7.3 log CFU/chip. At 12°C, the population of *E. coli* O157:H7 on the stainless steel surfaces was generally lower than at 22°C (Fig. 5.1). Chips placed in TSB had the lowest cell counts compared to the cell counts in 10% TSB and MSB. On day 3, the cell counts on chips grown in TSB decreased to 6.15 log CFU/chip at 22°C and to 6.90 log CFU/chip at 12°C. The total cell counts increased to 6.95 log CFU/g and 6.71 log CFU/g at 22°C and 12°C respectively on day 7 and thereafter remained unchanged for 21 days. For 10% TSB, the total cell counts on the chips increased from 7.30 log CFU/chip on day 0 to 7.70 log CFU/chip at 22°C and decreased to 7.08 at 12°C on day 3. On day 7, there was an increase in cell counts to 8.33 log CFU/chip at 22°C and to 7.19 log CFU/chip at 12°C. The cell populations remained at these levels with no significant changes for the duration of the 21-day incubation period.

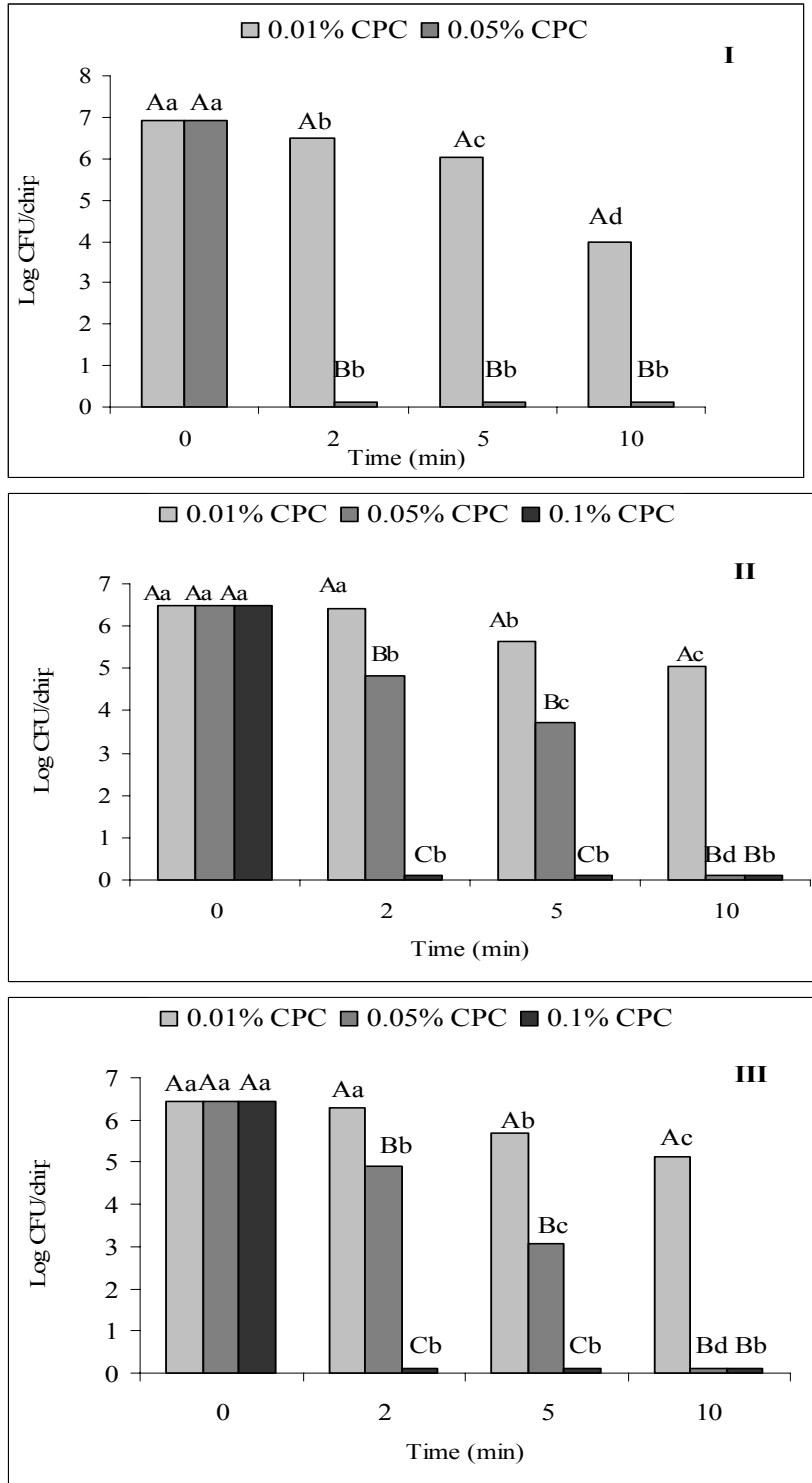
Chips placed in MSB had the highest cell counts compared to those grown in TSB or 10% TSB at both the temperatures. The total cell counts on the chips increased from 7.3 log CFU/chip initially to 8.10 and 7.79 log CFU/chip on day 3 at 22°C and 12°C respectively. On day 7, there was an increase in cell counts to 9.13 log CFU/chip on the chips incubated at 22°C and to 8.62 log CFU/chip on chips incubated at 12°C. The total



**Fig. 5.1. Total cell counts produced by *E. coli* O157:H7 on the surface of stainless steel chips immersed in TSB, 10% TSB and MSB at (I) 12°C and (II) 22°C. Within the temperature of incubation and incubation time, values not marked by the same letter for total cell counts are significantly different ( $P \leq 0.05$ ). Detection limits were 0.60 log CFU/chip.**



**Fig. 5.2. Inactivation of *E. coli* O157:H7 biofilms on the surface of stainless steel chips immersed in TSB at 22°C for (I) 2 days (II) 10 days and (III) 21 days.** Within the age of the biofilm and concentration of CPC, values not marked by the same capital letter for total cell counts are significantly different ( $P \leq 0.05$ ). Within the age of the biofilm and treatment time, values not marked by the same lowercase letter for total cell counts are significantly different ( $P \leq 0.05$ ). Detection limits were 0.60 log CFU/chip.



**Fig. 5.3. Inactivation of *E. coli* O157:H7 biofilms on the surface of stainless steel chips immersed in TSB at 12°C for (I) 2 days (II) 10 days and (III) 21 days.** Within the age of the biofilm and concentration of CPC, values not marked by the same capital letter for total cell counts are significantly different ( $P \leq 0.05$ ). Within the age of the biofilm and treatment time, values not marked by the same lowercase letter for total cell counts are significantly different ( $P \leq 0.05$ ). Detection limits were 0.60 log CFU/chip.

cell counts increased further to 9.46 and 9.06 log CFU/chip at 22°C and 12°C respectively by day 10. On day 21, the total cell counts were highest at 10.15 and 9.30 log CFU/chip at 22°C and 12°C respectively.

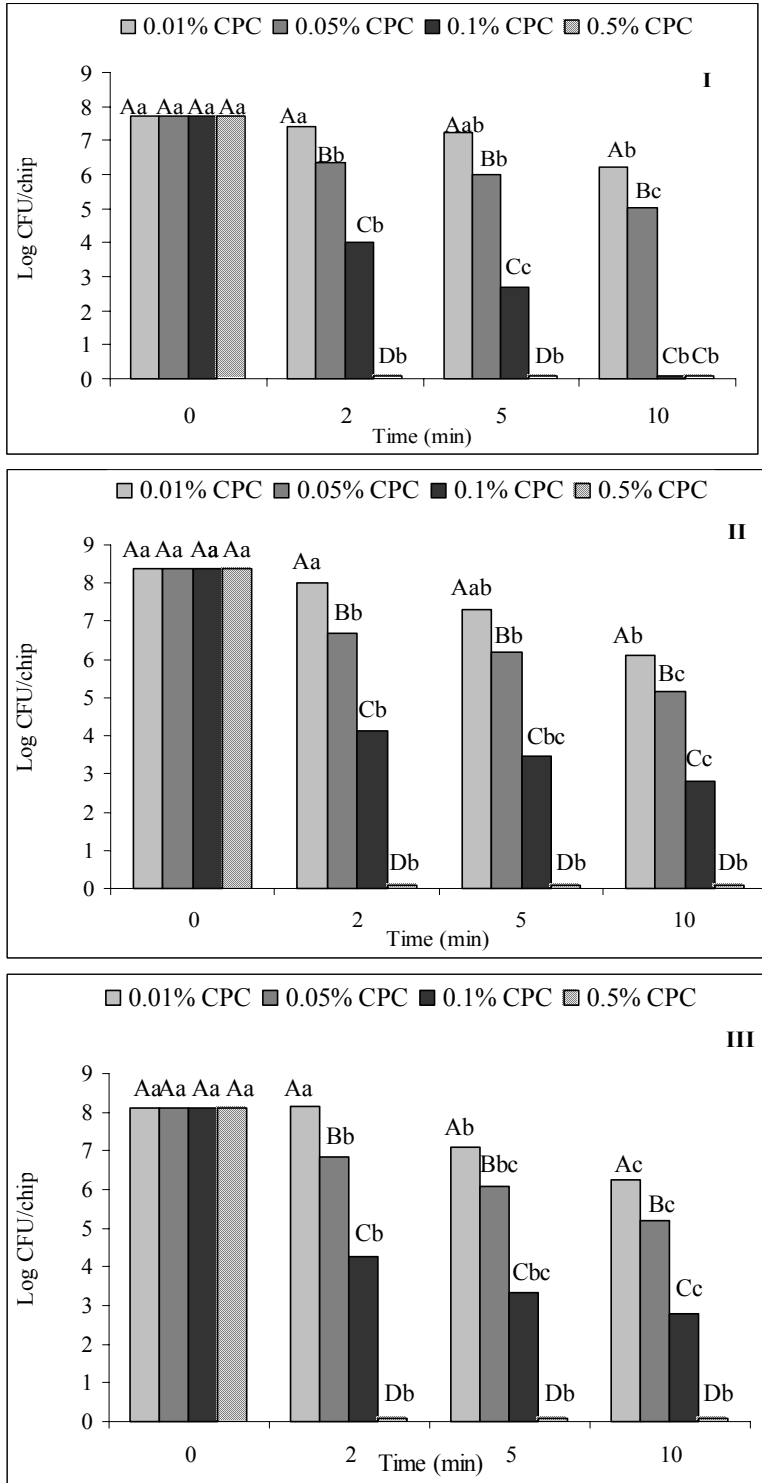
#### **CPC Inactivation of *E. coli* O157:H7 Biofilms Grown in TSB**

Treatment of 2-day biofilms at 22°C with 0.05% CPC resulted in highest reductions in total cell counts from 6.15 log CFU/chip initially to 4.26 and 3.18 log CFU/chip within 2 and 5 min respectively and nondetectable levels in 10 min. Whereas for 21-day biofilms, a higher concentration of CPC was required to attain non-detectable levels. Cell counts decreased from 7.10 log CFU/chip initially to 1.32 log CFU/chip in 2 min and non-detectable levels in 5 and 10 min with 0.1% CPC (Fig. 5.2).

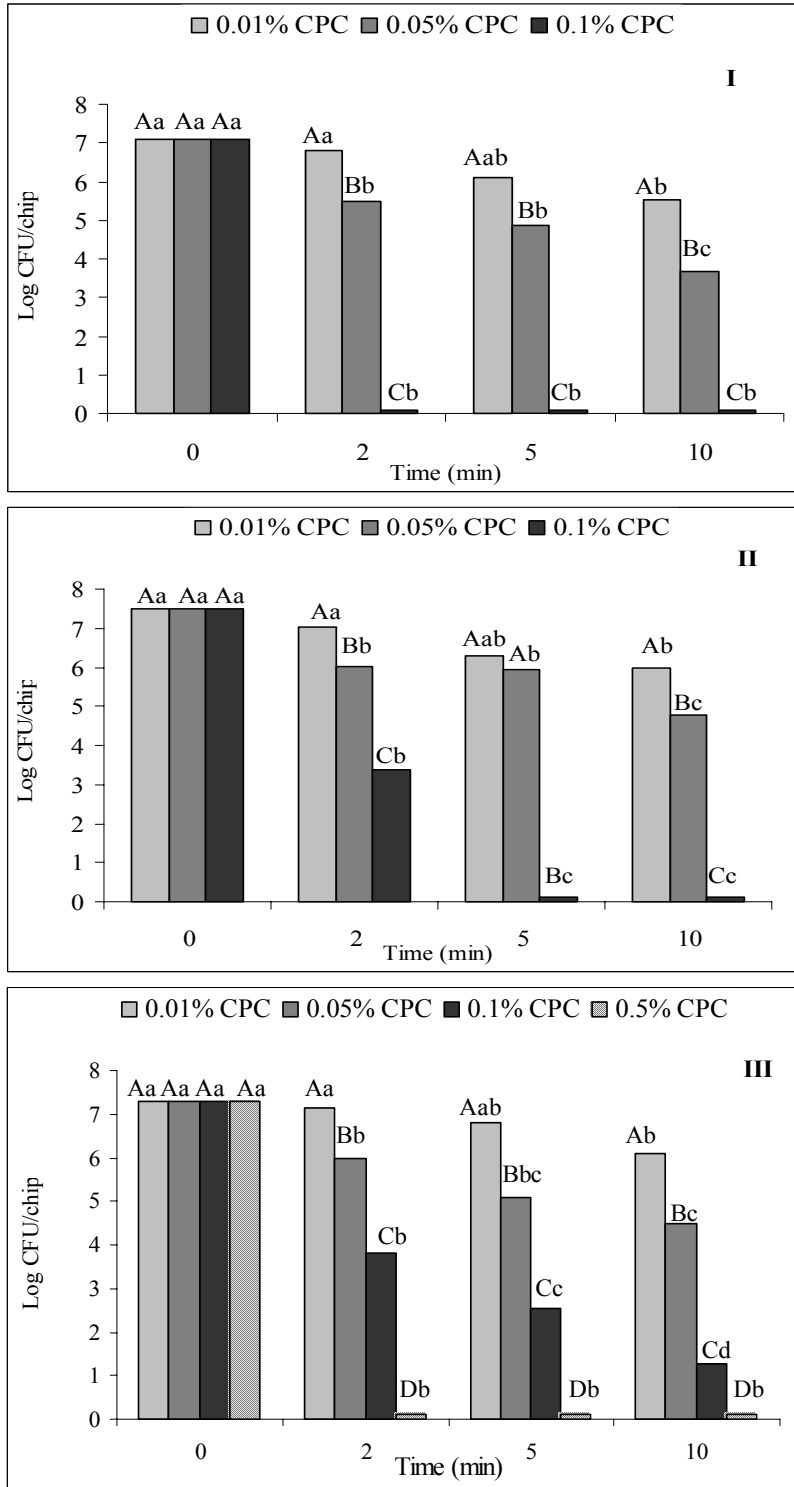
Inactivation of biofilms at 12°C occurred at lower CPC concentrations compared to biofilms grown at 22°C. Non-detectable levels were obtained after exposure with 0.05% within only 2 min, whereas for 21-day biofilms, non-detectable levels were observed with 0.05% CPC only after 10 min (Fig. 5.3)

#### **CPC Inactivation of *E. coli* O157:H7 Biofilms Grown in 10% TSB**

Treatment with 0.05% CPC resulted in highest reductions of the total cell counts, on 2-day biofilms at 22°C, from 7.70 log CFU/chip initially to 6.42, 6.04 and 5.01 log CFU/chip in 2, 5 and 10 min respectively. For 21 day old biofilms, maximum reductions occurred with 0.1% CPC from 8.10 log CFU/chip initially to 3.81, 2.54, and 1.27 log CFU/chip within 2, 5, and 10 min, respectively. Treatment with 0.1% and 0.5% CPC decreased cell counts to non-detectable levels for 2-day and 21-day biofilms, respectively (Fig. 5.4).



**Fig. 5.4. Inactivation of *E. coli* O157:H7 biofilms on the surface of stainless steel chips immersed in 10% TSB at 22°C for (I) 2 days (II) 10 days and (III) 21 days.** Within the age of the biofilm and concentration of CPC, values not marked by the same capital letter for total cell counts are significantly different ( $P \leq 0.05$ ). Within the age of the biofilm and treatment time, values not marked by the same lowercase letter for total cell counts are significantly different ( $P \leq 0.05$ ). Detection limits were 0.60 log CFU/chip.



**Fig. 5.5. Inactivation of *E. coli* O157:H7 biofilms on the surface of stainless steel chips immersed in 10% TSB at 12°C for (I) 2 days (II) 10 days and (III) 21 days.** Within the age of the biofilm and concentration of CPC, values not marked by the same capital letter for total cell counts are significantly different ( $P \leq 0.05$ ). Within the age of the biofilm and treatment time, values not marked by the same lowercase letter for total cell counts are significantly different ( $P \leq 0.05$ ). Detection limits were 0.60 log CFU/chip.

For 2-day biofilms at 12°C, treatment with 0.05% CPC resulted in maximum reductions in total cell counts from 7.10 log CFU/chip initially to 5.48, 4.88 and 3.66 log CFU/chip within 2, 5, and 10 min respectively and non-detectable levels with 0.1% CPC. For 21-day, non-detectable levels were observed using 0.5% CPC (Fig. 5.5).

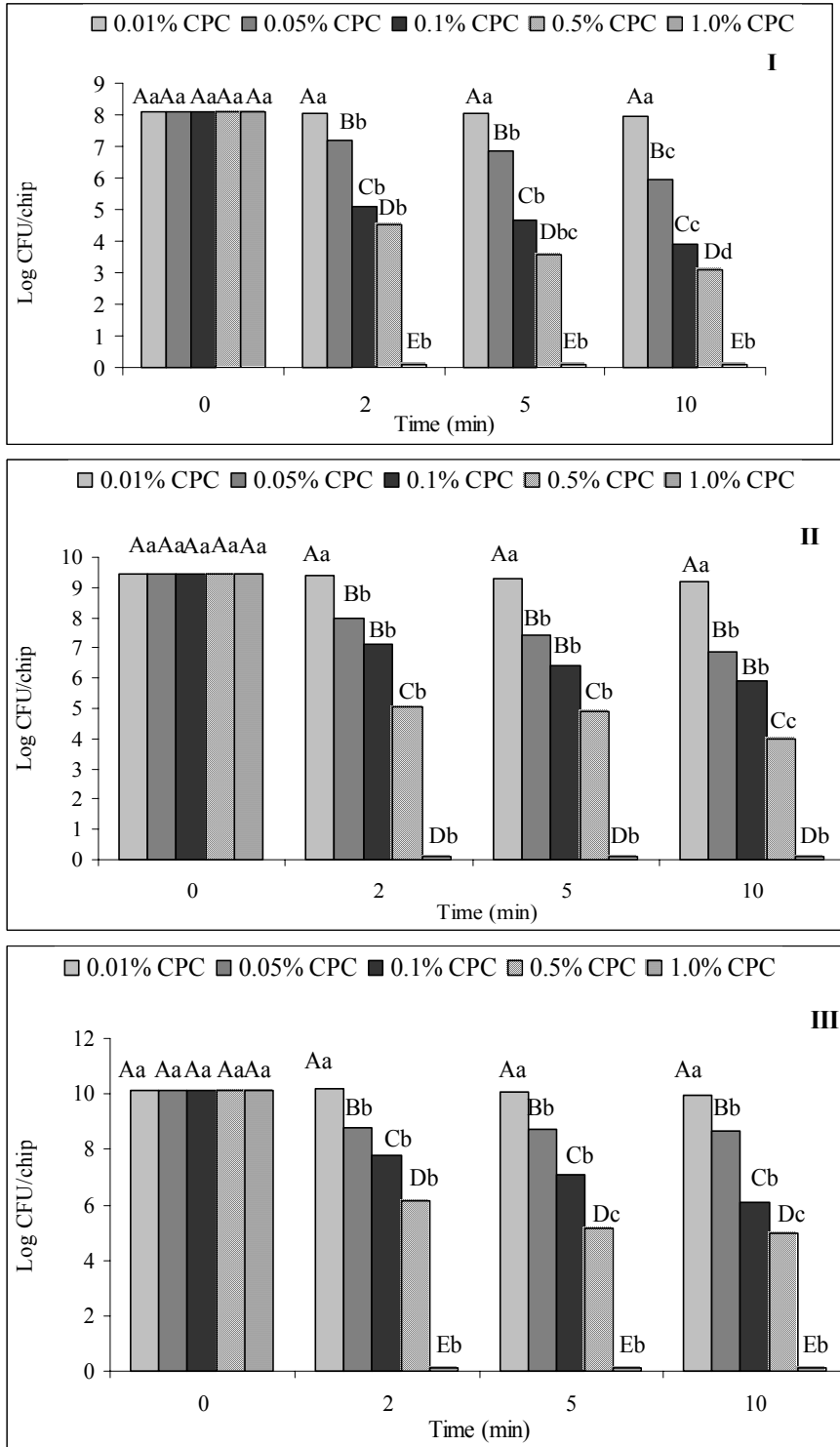
### **CPC Inactivation of *E. coli* O157:H7 Biofilms Grown in MSB**

Biofilms grown in MSB were less sensitive to CPC compared to those grown in TSB and 10% TSB (Fig. 5.6). For 2-day biofilms at 22°C, highest reductions occurred with 0.5% CPC resulting in a decrease in total cell counts from 8.10 log CFU/chip initially to 4.53, 3.59 and 3.08 log CFU/chip within 2, 5, and 10 min, respectively. Lower reductions were obtained with the 21-day biofilm treated with 0.5% CPC. Cell counts decreased to 6.17, 5.16, and 4.96 log CFU/chip in 2, 5, and 10 min, respectively when treated with 0.5% CPC compared to 10.14 log CFU/chip without CPC treatment. Non-detectable levels were obtained with 1.0 % CPC for both 2-day and 21-day biofilms.

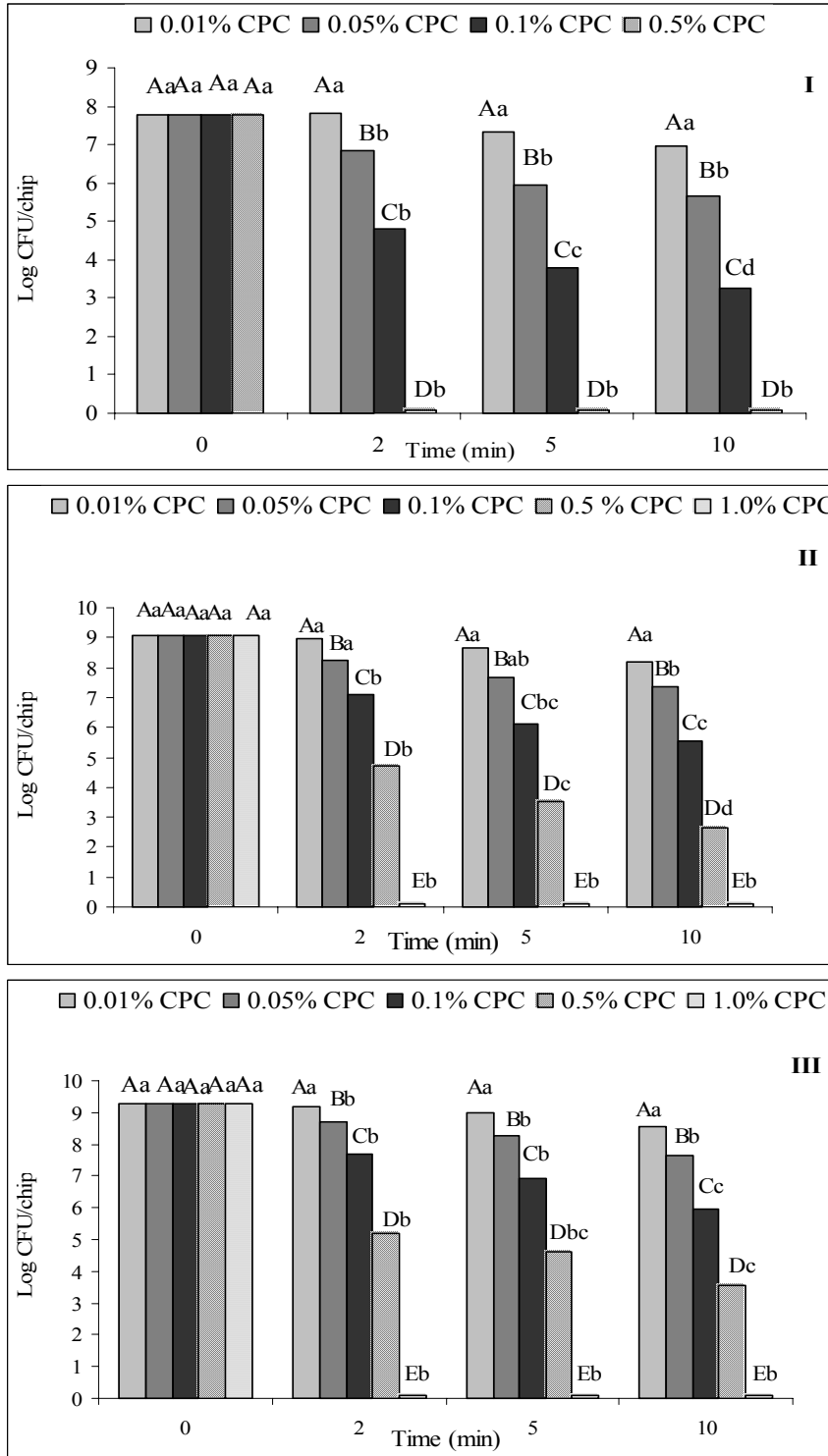
Lower concentrations of CPC were effective at reducing *E. coli* O157:H7 biofilms grown at 12°C compared to biofilms grown at 22°C (Fig. 5.7). At 0.5% CPC, *E. coli* O157:H7 biofilms were totally inactivated and reached non-detectable levels within 2 min for 2-day biofilm, whereas non-detectable for 21-day biofilms were reached only with 1.0% CPC in 2 min.

## **DISCUSSION**

The purpose of this study was to determine the antimicrobial efficacy of CPC on *E. coli* O157:H7 biofilms grown in three different culture media on stainless steel chips at two temperatures. Disinfection studies were performed using various levels of CPC to compare the effects of concentration and time of exposure on bacterial cells adhering to



**Fig. 5.6. Inactivation of *E. coli* O157:H7 biofilms on the surface of stainless steel chips immersed in MSB at 22°C for (I) 2 days (II) 10 days and (III) 21 days.** Within the age of the biofilm and concentration of CPC, values not marked by the same capital letter for total cell counts are significantly different ( $P \leq 0.05$ ). Within the age of the biofilm and treatment time, values not marked by the same lowercase letter for total cell counts are significantly different ( $P \leq 0.05$ ). Detection limits were 0.60 log CFU/chip.



**Fig. 5.7. Inactivation of *E. coli* O157:H7 biofilms on the surface of stainless steel chips immersed in MSB at 12°C for (I) 2 days (II) 10 days and (III) 21 days.** Within the age of the biofilm and concentration of CPC, values not marked by the same capital letter for total cell counts are significantly different ( $P \leq 0.05$ ). Within the age of the biofilm and treatment time, values not marked by the same lowercase letter for total cell counts are significantly different ( $P \leq 0.05$ ). Detection limits were 0.60 log CFU/chip.

stainless steel surfaces. Biofilms grown in MSB were more resistant to CPC than biofilms grown in TSB or 10% TSB. In general, the inactivation of biofilms grown in all the three media were time and concentration dependent. Longer exposure times significantly increased biocidal activity and old biofilms were more resistant to CPC sanitizing than young biofilms. There were significant reductions in total cell counts between successive treatment times at 2, 5, or 10 min compared to the control and between each CPC concentration.

Beef and beef products have been most frequently implicated in outbreaks of foodborne *E. coli* O157 infection. Contamination occurs when pathogens from animal feces, hide, or the intestine are transferred to the muscle tissue during processing. *E. coli* O157:H7 biofilms were grown on stainless steel chips, in the present study, since it is the most commonly used metal in food processing facilities because it has poor adhesion characteristics and good cleanability (Lelieveld et al.,2005). However, as shown in this study and several others, *E. coli* O157:H7 can survive and produce biofilms on stainless steel and on a variety of metal surfaces indicating a potential for cross-contamination (Ryu et al., 2004; Wilks et al., 2005).

Bacteria, including *E. coli* O157:H7, in biofilms are generally more resistant to environmental stresses such as sanitizers than organisms in suspension and, therefore, suspension tests give no indication of the susceptibility of microorganisms in actual conditions (Foschino et al.,1998; Ryu and Beuchat, 2004). Suspension studies were not conducted in our study because they do not imitate the growth conditions on surfaces, which require much higher concentrations of sanitizers to deactivate biofilms.

Bacterial attachment to inert surfaces is influenced by the attachment media as well as by other factors such as growth phase of cells, properties of the inert material, presence of organic material, environmental pH and temperature (Kumar and Anand, 1998). Results from this study showed that type of growth medium exerted a major influence on biofilm accumulation; biofilm development was greatest on chips grown in MSB as compared to TSB or 10% TSB. Visible slime was evident on chips growing in MSB indicating production of large amounts of exopolymeric substances by *E. coli* O157:H7 as it was forming the biofilm. However, slime production was not visible for chips grown in TSB or 10% TSB. Dewanti and Wong (1995) studied the influence of culture media on the *E. coli* O157:H7 biofilm formation. They studied the development of biofilms on stainless steel chips in TSB, 10% TSB, 0.1% Bacto Peptone, and MSB. They reported that *E. coli* O157:H7 developed biofilms in all media, but a higher number of cells were detected in the biofilm when the organism was grown in low-nutrient media. They hypothesized that the adsorption of proteins present in TSB to inert surfaces can decrease the attachment of bacteria compared to MSB which has very little or no proteins. Similar results were obtained by Ryu and Beuchat (2004) who demonstrated that low nutrient availability favored production of EPS by *E. coli* O157:H7. It is believed that the growth media might influence cell surface chemistry by stimulating extracellular polymer production or by causing changes in other surface structures.

The attachment of *E. coli* O157:H7 to stainless steel chips was time-dependent. Thus the adhesion of *E. coli* O157:H7 cells to stainless steel surfaces showed increases in cell numbers with longer exposures on chips growing in MSB, and less significantly on chips growing in 10% TSB. However chips growing in TSB had reduced attachment rates

on day 21 compared to day 0. Similar results were observed by Dewanti and Wong (1995) who found that the number of *E. coli* O157:H7 cells attached to stainless steel chips after 1 h in TSB was significantly lower than that those attached to stainless steel in MSM supplemented with 0.04% glucose.

Temperature can also effect attachment. It was observed from this study that biofilm development on the surface of stainless steel is, in general, more favorable at 22°C than at 12°C. However, biofilm formation was reduced but not eliminated by growth at low temperatures. It has been reported that cells usually attach better at temperatures supporting growth than at refrigeration temperature. Herald and Zottola (1988) observed that 21°C was more favorable than 35°C or 10°C for attachment of *Yersinia* to stainless steel surfaces. *Listeria* strains have been reported to adhere to stainless steel in significantly greater numbers at 18°C than at 4°C. They concluded that adhesion was directly related to flagellar movement and the production of exopolymers at favorable temperatures.

Quaternary ammonium compounds (QAC) such as CPC are hydrophilic cationic molecules that readily adsorb to negatively charged bacterial surface, penetrate the cell wall and disrupt the cytoplasmic membrane. Cells embedded in a biofilm are enveloped by EPS that prevents penetration and protects from QAC (Sakagami et al., 1989). The inactivation of *E. coli* O157:H7 biofilms grown in all the three media on exposure to CPC were time and concentration dependent. Longer exposure times significantly increased biocidal activity. For all of the concentrations of CPC used, a 10 minute contact time was more effective than a 5 minute or a 2 minute contact time. This is an important consideration for the application of sanitizers in the food industry.

Also, 21-day and 10-day biofilms were not more resistant to sanitizing than 2-day biofilms. This finding is in agreement with previous studies showing increased resistance with biofilm age (LeChevallier et al., 1988). The fact that the younger biofilms were more sensitive to CPC than old biofilms can be explained in two ways. Firstly, exponentially growing cells in younger biofilms are more sensitive to environmental stresses than are stationary phase cells in old biofilms because of the high energy demand and intense regulation of growth components associated with actively growing cells. Secondly, the increasing numbers of attached cells surrounded by EPS in older biofilms confer protection against the effects of sanitizers by preventing the penetration of the sanitizer into the inner layers of the biofilm (Costerton et al., 1999).

CPC was more effective in inactivating biofilms grown in TSB and 10% TSB compared to MSB grown biofilms. Exposure to CPC induced a similar reduction in viable counts of biofilms grown in TSB and 10% TSB. Concentrations of 0.05% and 0.1% were sufficient to completely inactivate biofilms (2-day, 10-day, and 21-day biofilms) grown in TSB and 10% TSB respectively. Whereas, a concentration of 1.0% was required to completely inactivate 21-day biofilms grown in MSB. Previous studies have shown that the susceptibility of bacteria to antimicrobial agents is highly influenced by conditions used in the cultivation of the organisms (Anwar et al., 1990). Visible slime was evident on chips growing in MSB indicating production of large amounts of exopolymeric substances *E. coli* O157:H7 as it formed a biofilm. The reduced susceptibility of biofilms grown in MSB is probably because the exopolysaccharide (EPS) could consume the antimicrobial agent, either by adsorption or reaction, and protect bacteria in the deeper layers. Samrakandi et al. (1997) compared the biocidal

activity of antimicrobial agents against biofilms grown in different media and differ in their EPS content. Their results showed that culture conditions significantly affected EPS production and biofilm composition, with consequent modification of biocide efficacy on the biofilms.

The results from this study show that antimicrobial activity of CPC can be significantly affected by culture media used to grow biofilms. This study also suggests that *E. coli* O157:H7 biofilms grown under low-nutrient conditions were more resistant than biofilms grown in rich media. It is therefore necessary to test antimicrobial activity under conditions similar to those found in food processing environments. Further studies are required to test efficacy of CPC in presence of organic matter because some sanitizers are inactivated in presence of organic material.

Cleaning and disinfecting agents used on food processing surfaces can cause taints, or can become, potentially, hazardous and toxic if they leave residues on surfaces. It has been estimated that up to 30% of food taint complaints are associated with cleaning and disinfecting chemicals, producing taints that can enter food products accidentally, for example from poor rinsing or aerial transfer or from 'no rinse' disinfectants designed to be left on surfaces to provide more lasting protection against recontamination (Lelieveld et al., 2005). QACs are commonly used as sanitizers in the food industry. Typical concentrations for biocidal effectiveness of these quaternary compounds range from about 0.001% to about 0.8% (Lelieveld et al., 2005). CPC is a QAC that is currently only approved for use in poultry processing by the FDA. Since CPC has been shown to be safe to be used on foods and has also been demonstrated to be environmentally safe in many aspects, it can be used to effectively control *E. coli* O157:H7 biofilms on food contact

surfaces. However, further studies are required to test the efficacy of CPC in presence of organic matter and also on mixed species biofilms.

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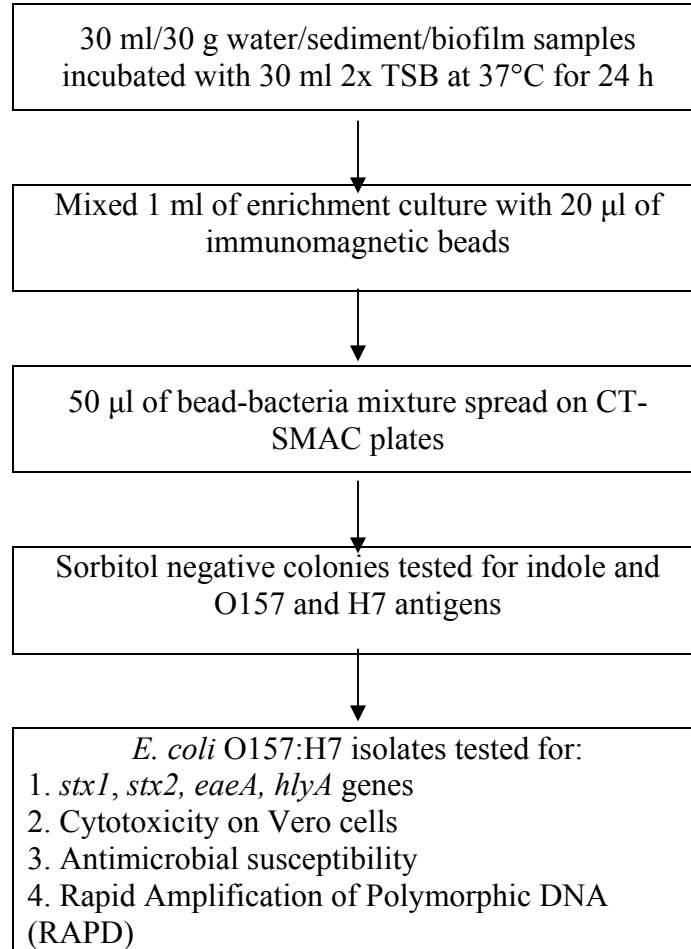
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**CHAPTER 6**  
**CONCLUSION**

Results from this study showed an *E. coli* O157:H7 prevalence of 4.5% in the water troughs of the four different geographically located cattle farms in Louisiana. The one-time sampling in the present study did not allow the evaluation of persistence, seasonal variation, or geographical differences in prevalence of *E. coli* O157:H7 in the cattle farms examined in this study. Temporal studies are required to evaluate the above issues and to determine any potential relationship between *E. coli* O157:H7 persistence in the cattle water troughs and in the cattle feces. All the five strains of *E. coli* O157:H7 used in the survival study showed variability in the growth at different temperatures. Higher temperatures (25°C and 37°C) resulted in greater decrease of *E. coli* O157:H7 than at lower temperatures (5°C and 15°C), especially in the presence of natural microflora. It was also observed that the growth and survival of human *E. coli* O157:H7 isolates was significantly lower than environmental isolates at all temperatures. Overall, most of the isolates were able to survive for 60 days at all temperatures both in the presence and absence of competing microflora. The biofilm study showed that *E. coli* O157:H7 growth in biofilms was culture and temperature dependent. Biofilms grown in MSB were more resistant to CPC than biofilms grown in TSB or 10% TSB. In general, the inactivation of biofilms grown in all the three media were time and concentration dependent. On the whole, <1.0% CPC was effective in inactivation of *E. coli* O157:H7 biofilms grown on stainless steel in all three culture conditions.

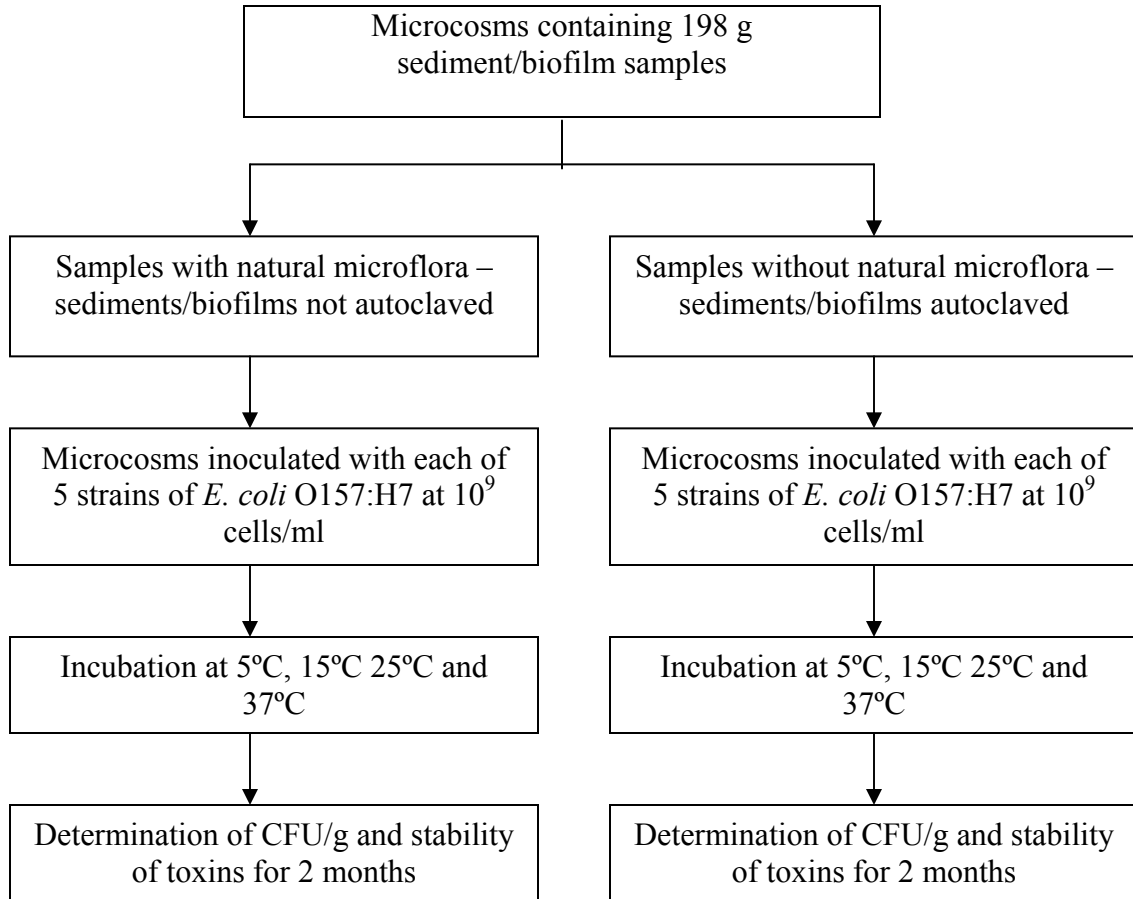
## APPENDIX 1

### FLOW CHART FOR DETECTION OF *E. COLI* O157:H7 IN CATTLE WATER TROUGHS



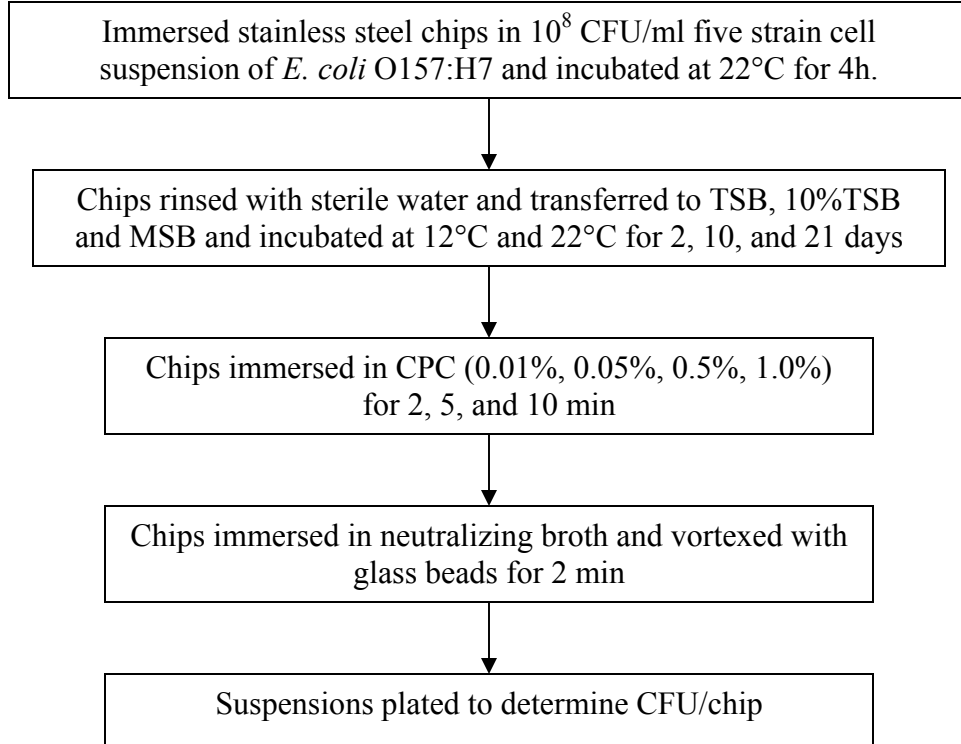
## APPENDIX 2

### FLOW CHART FOR SURVIVAL STUDIES OF *E. COLI* O157:H7 IN CATTLE WATER TROUGHS



### APPENDIX 3

#### FLOW CHART FOR EFFECT OF CPC TREATMENT AGAINST *E. COLI* O157:H7 BIOFILMS ON THE SURFACE OF STAINLESS STEEL



## VITA

The author was born in Hyderabad, India, where she got her high school education. She received a Bachelor of Science degree in biology and a Master of Science degree in botany from Osmania University, Hyderabad. She received a second Master of Science degree in environmental sciences from Louisiana State University in August 2003. She started her doctoral program in food science at Louisiana State University in August 2003 and will receive this degree in December 2006.