ABSTRACT

SKINNER, BRANDON MICHAEL. *Escherichia coli* O157:H7 Biofilm Formation and Susceptibility to Disinfectants *In Vitro* and in Footbaths. (Under the direction of Drs. Megan Jacob and Sophia Kathariou).

Escherichia coli O157:H7 is a human pathogen that can cause severe gastrointestinal and systemic disease. Ruminants are the natural, asymptomatic reservoir for *E. coli* O157:H7, which may spread through the food chain or by direct contact with contaminated feces. *Escherichia coli* O157:H7 outbreaks have been reported at agritourism events including petting zoos, fairs, and livestock interactions. Footbath use at agritourism events may serve as one tool to control *E. coli* O157:H7 transmission; however, specific recommendations to optimize efficacy based on the type of disinfectant, the amount of organic matter, environmental conditions, and the potential of the bacteria to form biofilms on footbaths are not available. Our objectives were to determine the biofilm-forming potential of *E. coli* O157:H7 strains and the susceptibility of those strains towards commonly used footbath disinfectants both *in vitro* and within simulated footbaths.

Seven genetically distinct *E. coli* O157:H7 strains from North Carolina meat goats were evaluated for their ability to form biofilms on polystyrene pegs and rubber footbath surfaces at different incubation times and temperatures. Crystal violet assays were performed to determine biofilm biomass, and the biofilms were sonicated to establish the concentration of viable bacteria within the biofilms. Variations in biofilm density were observed between isolates, incubation times, and incubation temperatures on polystyrene plastic, with a significant three-way interaction ($p < 0.0001$). Longer incubation times and lower temperatures generally favoring higher biofilm densities. A significant two-way interaction between strains and temperatures was observed on rubber footbaths ($p = 0.0233$). The most

dense biofilms were formed at lower temperatures (22˚C), though these biofilms likely were not mature. Further work to characterize the antimicrobial and disinfectant tolerance of *E. coli* O157:H7 in an immature biofilm may be warranted.

Additionally, the minimum inhibitory concentrations (MIC) of commonly-used laboratory-grade disinfectant compounds towards *E. coli* O157:H7 strains were determined. The median MICs were 20 ppm hydrogen peroxide, 320 ppm sodium hypochlorite, 625 ppm glutaraldehyde, 3.2 ppm didecyl-dimethyl ammonium chloride (DDAC), and 2500 ppm phenol, with little variability between isolates. Time-kill assay performed *in vitro* using laboratory-grade disinfectants at 0.5 × MIC, 1 × MIC, and 2 × MIC. At 2 × MIC showed that hydrogen peroxide and phenol reduced the *E. coli* O157:H7 concentration by 3 $\log_{10}$(CFU/mL) by 4 hours after contact, while sodium hypochlorite and DDAC killed the *E. coli* O157:H7 within 20 minutes, and glutaraldehyde killed the bacteria on contact. Time-kill assays were also performed within simulated footbaths with and without added organic matter contamination using commercially available disinfectants (0.26% Clorox® bleach, 1% Virkon™ S, and 0.034% Virex® II 256). All three commercial disinfectants killed the *E. coli* O157:H7 on contact in the absence of organic contamination, but viable *E. coli* O157:H7 was recovered from the organically contaminated footbaths with Clorox® bleach and Virex® II 256 throughout the course of the assays. No *E. coli* O157:H7 was recovered from the Virkon™ S footbaths at or after 10 minutes of exposure. Our findings highlight the importance of choosing appropriate footbath disinfectants and regularly cleaning footbaths to prevent persistence of *E. coli* O157:H7 within the footbaths and to maintain the efficacy of their use.

Escherichia coli O157:H7 Biofilm Formation and Susceptibility to Disinfectants *In Vitro* and in Footbaths

by
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DEDICATION

To my mother, whose endless love and support made this journey possible.

BIOGRAPHY

Brandon Skinner was born and raised in Wilson, North Carolina. As a child, he always had a fascination with science, and he wanted to grow up to become a doctor. He was accepted into the first graduating class of Wilson Early College Academy, where he received a high school diploma and an Associate's degree in four years. He attended Barton College, a small private college in Wilson, North Carolina. It was here that he discovered his interest in scientific research. Under the guidance of several fantastic professors, he completed undergraduate research focusing on both biology and analytical chemistry.

After earning his Bachelor of Science in Biology, Brandon enrolled in and attended North Carolina State University to pursue a graduate degree in Microbiology. Originally accepted as a PhD student, he later realized that a Master of Science degree better fit his career goals and interests. He joined Dr. Megan Jacob's lab in the Spring of 2017, where he pursued research in *E. coli* O157:H7 biofilm formation and disinfectant susceptibility. He has presented his work at the Annual College of Veterinary Medicine Research Forum, the 2017 North Carolina American Society for Microbiology meeting, and the 98th Annual Conference of Research Workers in Animal Diseases, where he won the award for best student presentation in Biosafety and Biosecurity.

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CHAPTER 1

Biofilm Potential of *Escherichia coli* O157:H7 and its Susceptibility to Disinfectants: A Review of Current Literature

Abstract

Human *Escherichia coli* O157:H7 infections are associated with gastrointestinal and occasionally systemic disease. The ruminant gastrointestinal tract is the natural reservoir for *E. coli* O157:H7, where it can be shed asymptomatically in the feces. Transmission of the pathogen to people is via the fecal-oral route, through contaminated food, water, or after direct contact with livestock. The use of disinfectants in food processing or at livestock interactions are one *E. coli* O157:H7 reduction strategy. Factors including the chemical compound, disinfectant concentration, environmental conditions, and presence of organic matter are important considerations to optimize efficacy. An additional consideration is the ability of *E. coli* O157:H7 to form biofilm, which may be associated with tolerance to disinfecting compounds. Even at recommended concentrations, many disinfectants may not adequately reduce *E. coli* O157:H7 in biofilms to prevent future transmission of the organism. This review aims to summarize the current research associated with *E. coli* O157:H7 biofilm formation and the pathogen's susceptibility to disinfectants in planktonic form and in mature biofilms.

1.1 Introduction

First identified as a pathogen in 1982, *Escherichia coli* O157:H7 is associated with foodborne diseases, including bloody diarrhea, thrombotic thrombocytopenic purpura, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Doyle, 1991; Mead and Griffin, 1998; Wang *et al*., 2012). Hemolytic uremic syndrome is a life-threatening complication that can lead to kidney failure, particularly in young children (Durso, 2005). *Escherichia coli* O157:H7 is estimated to cause over 96,000 illness each year, resulting in over 3,000 hospitalizations and 30 deaths (Scallan *et al*., 2011). Outbreaks of *E. coli* O157:H7 have been associated with consumption of undercooked ground beef or contaminated dairy products (Hrudey *et al*., 2003; Hu, Zhang, and Meitzler, 1999; Kassenborg *et al*., 2004), contaminated produce (Centers for Disease Control and Prevention, 2018), and from contact with animals at livestock interaction events (Centers for Disease Control and Prevention, 2006; Laughlin *et al*., 2016). The most cost-effective way to control *E. coli* O157:H7 infections is to prevent the infection from occurring, leading to the development of many mitigation and intervention strategies through the food production chain and in areas where the public is in direct contact with livestock.

The biofilm-forming potential of *E. coli* O157:H7 is of increasing recognition and concern to controlling transmission of the pathogen in food processing and farm animal environments (Srey, Jahid, and Ha, 2013; Van Houdt and Michiels, 2010; Bridier *et al*., 2011; Shi and Zhu, 2009; Hancock, 1997). A biofilm is defined as a collective group of bacteria of the same or different species that are enclosed in a self-protective extracellular matrix (ECM) and have altered gene transcription and a different growth rate than planktonic cells (Saur, Rickard, and Davies, 2007; Donlan and Costerton, 2002). Many pathogenic

bacteria are capable of forming biofilms on a variety of surfaces (Shi and Zhu, 2009). In food processing and farm environments, organic matter such as food scraps, wastewater, and feces often contaminate various surfaces, producing ideal places for microorganisms to grow and form biofilms. The attachment and development of bacterial biofilms can be a potential source of contamination that can lead to transmission of disease (Dewanti and Wong, 1995; Chmielewski and Frank, 2003). Once a biofilm has formed on a surface, it is difficult to eliminate because the bacterial cells are protected by their ECM (Chmielewski and Frank, 2003). Even when equipment is cleaned and sanitized using good manufacturing practices, microorganisms may still remain on equipment surfaces (Maxcy, 1964, Czechowski, 1990, Mattila, 1990). Studies have shown that bacteria in biofilms are more likely to survive sanitation measures than planktonic bacteria (Mah and O'Toole, 2001; Mosteller and Bishop, 1993; Wang *et al*., 2014). Some have even suggested that the working concentrations of disinfectants should be increased 10- or 100-fold to effectively inactivate bacteria in biofilms (Sofos and Geornaras, 2010).

Several *E. coli* O157:H7 strains have been shown to attach to, colonize, and form biofilm on food contact surfaces, meat products, vegetables, and other produce, and they may survive on these surfaces for prolonged periods of time depending on the environmental conditions (Marouani-Gardi, Augier, and Carpentier, 2009; Silagyi *et al.* 2009; Maxcy, 1971; Torres *et al*, 2005; Ryu, Kim, and Beuchat, 2004). The focus of this review is to summarize the body of literature that characterizes the biofilm-forming potential of *E. coli* O157:H7 on different of surfaces, as well as the efficacy of disinfectants to inactivate *E. coli* O157:H7 in suspension, on surfaces of foods, and in biofilms.

1.2 Biofilm Formation of *E. coli* O157:H7

Many bacteria, including *E. coli* O157:H7, may form biofilms on surfaces by embedding themselves in a polymer matrix (Characklis and Marshall, 1990). Biofilm production is typically strain-dependent in *E. coli* O157:H7, and environmental factors such as temperature and nutrient availability may affect biofilm formation (Mendonca *et al*., 2012; Dewanti and Wong, 1995; Ulich *et al*., 2014). Biofilms present a challenge for control of transmission in food processing or livestock handling environments. *Escherichia coli* O157:H7 has previously been isolated from biofilms on watering troughs in animal enclosures (Hancock *et al.*, 1998; Crump *et al.*, 2002). Portions of microbially-inhabited biofilms may detach and colonize elsewhere (Dewanti and Wong, 1995). Additionally, bacteria embedded in biofilms are hardier than their planktonic counterparts because the ECM offers protection from desiccation, extreme temperatures, and the presence of chemical disinfectants (Costerton *et al*., 1987; Nickel, 1990; Marrie and Costerton, 1981; Stickler *et al*., 1989; Wang, 2014). The formation of biofilms by *E. coli* O157:H7 is dependent on several elements including adhesion factors and environmental conditions.

1.2.1 Adhesion Factors

Adhesion factors are important for the attachment and biofilm production of *E. coli* O157:H7 on organic and abiotic surfaces. These adhesion factors include curli fimbriae, flagella, cellulose, lipopolysaccharides (LPS), colonic acid, and several outer membrane proteins (Carter *et al*., 2016; Cookson, Cooley, and Woodard, 2002; Danese, Pratt, and Kolter, 2000; Prigent-Combaret *et al*., 2000; Ryu and Beuchat, 2005; Saldana *et al*., 2009). A study conducted by Biscola *et al.* (2011) evaluated the biofilm potential of Shiga toxin-

producing *E. coli* (STEC) strains and determined the possibility of a link between biofilm formation and the presence and expression of adhesin genes. They reported 28% of the *E. coli* O157:H7 strains and 51% of the non-O157 STEC strains from different sources formed biofilms, and a strong correlation was noted between biofilm formation and the expression of curli and cellulose among the *E. coli* O157:H7 strains (Biscola, Abe, and Guth, 2011). Interestingly, the *fimH* gene for the expression of type 1 fimbriae was carried by all of the *E. coli* O157:H7 strains, but none of them produced type 1 fimbriae (Biscola, Abe, and Guth, 2011). This is likely due to the fact that *E. coli* O157:H7 has been shown to lack the ability to express type 1 fimbriae due to a deletion within the regulatory region that controls its expression (Li, Koch, and Cebula, 1997). Therefore, it is possible for bacteria to carry genes commonly involved in biofilm, but still lack biofilm-forming potential due to other genetic factors.

Poly-β-1,6-*N*-Acetyl-D-Glucosamine (PGA) is an extracellular polysaccharide that is important for biofilm formation in *E. coli* O157:H7. Encoded by the *pgaABCD* operon, which is ubiquitous in *E. coli*, PgaC functions as a glycosyltransferase to synthesize PGA, while PgaA exports the polysaccharide to the ECM (Itoh *et al*., 2008; Wang, Preston III, and Romeo, 2004). Once outside the cell, PGA functions as an adhesin to promote the structural stability of biofilms (Itoh *et al*., 2008). Previous work in *E. coli* K-12 has shown that all of the gene products from the *pgaABCD* operon are necessary for optimal biofilm formation (Itoh *et al*., 2008; Wang, Preston III, and Romeo, 2004)

Curli fimbriae are commonly studied as an important adhesion factor for *E. coli* O157:H7 biofilms. Curli are involved in the attachment of enteric pathogens to abiotic surfaces or plant tissues, but their expression is often strain-dependent, due in part to a

complex regulatory network that controls curli production (Carter *et al*., 2016). Carter *et al.* (2016) found that, in two *E. coli* O157:H7 outbreak strains, curli significantly enhanced initial attachment to spinach leaves and stainless steel surfaces by 5-fold. Curli was also required for biofilm formation on stainless steel, and it enhanced biofilm formation on glass by 19- to 27-fold (Carter *et al*., 2016). Some studies have suggested that curli expression is uncommon in *E. coli* O157:H7, but can occur in some strains under specific conditions (Uhlich, Keen, and Elder, 2001). Components of curli fimbriae are encoded by the *csgBAC* and *csgDEFG* operons, with *csgA* encoding the major structural subunit of curli (CsgA), and *csgD* encoding the transcriptional regulator of the *csgBAC* operon (CsgD) (Hammar *et al*., 1995; Romling, 2005; Barnhart and Chapman, 2006). Both *csgA* and *csgD* are needed for expression of curli fimbriae (Barnhart and Chapman, 2006). *E. coli* O157:H7 isolates with mutations in the *csgD* promoter have been linked to increased curli production due to upregulation in the transcription of *csgA* (Uhlich, Keen, and Elder, 2001).

Biscola *et al.* (2011) found that the curli gene sequences *csgA* and *crl* were carried by all the STEC strains that they isolated, but expression of these fimbriae only occurred in 28% of the *E. coli* O157:H7 strains. Additionally, studies have suggested that temperatures below 30°C promote curli gene expression (Arnqvist *et al*., 1992; Barnhart and Chapman, 2006). Curli and other adhesion factors appear to be important in the ability of *E. coli* O157:H7 to form biofilm on surfaces, and their expression and control should be further studied to expand the understanding of biofilm formation of *E. coli* O157:H7 and to identify therapeutic targets.

1.2.2 Environmental Conditions

Escherichia coli O157:H7 not only needs to be equipped with biofilm-forming genetic elements (including *csgBAC, csgDEFG, crl,* and *pgaABCD*) to form biofilm, but the appropriate environmental conditions must also be met. Different environmental conditions, such as temperature and nutrient availability, have been shown to significantly affect the biofilm-forming potential of *E. coli* O157:H7 (Biscola, Abe, and Guth, 2011; Carter *et al*., 2016; Dourou *et al*., 2011). Studies suggest that *E. coli* O157:H7 is capable of forming biofilm in a strain-dependent manner on plastic, stainless steel, glass, and organic surfaces such as lettuce, alfalfa sprouts, and epithelial cells (Wang *et al.*, 2016; Bae, Baek, and Lee, 2012; Boyer *et al*., 2007; Visvalinam and Holley, 2012; Torres *et al.*, 2005).

Previous work by Naves *et al.* (2008) found that clinical isolates of *E. coli* express different biofilm formation patterns on static assays depending on the strain and the culture medium. In their study, four clinical *E. coli* isolates were grown in minimal media and rich media, but strong and moderate biofilm formation only occurred in minimal media (Naves *et al.* 2008). This is consistent with a study by Dewanti and Wong (1995), who found that *E. coli* O157:H7 produced biofilms faster and with a higher number of adherent cells on stainless steel chips when the organisms were grown in minimal salts medium (MSM) as compared to trypticase soy broth (TSB). Biofilms developed in MSM also contained shorter bacterial cells and a thicker ECM (Dewanti and Wong, 1995).

Additionally, Wang *et al.* found that time variations can affect the outcome of biofilm assays. Biofilm assays of several strains of *E. coli* O157:H7 in LB-Low Salt (LB-LS) broth at room temperature showed most (n = 9 of 10) *E. coli* O157:H7 produced substantial biofilms

after 72 hours of growth, fewer (n = 2) produced substantial biofilms after 48 hours of growth, but none produced biofilm after just 24 hours of growth (Wang *et al*., 2012).

These studies demonstrate that environmental factors should be taken into consideration when interpreting biofilm formation by *E. coli* O157:H7 since variation in the media, time, and temperature can alter the results. In a practical setting, these studies show that under the right conditions, *E. coli* O157:H7 can produce biofilm on a variety of surfaces, including surfaces involved in food production or in livestock habitats. Biofilm formation by *E. coli* O157:H7 may increase tolerance of the pathogen to disinfectants, thus making it difficult to fully disinfect surfaces and prevent the spread of *E. coli* O157:H7.

1.3 Susceptibility of Planktonic *E. coli* O157:H7 to Disinfectants

1.3.1 Quaternary Ammonium-Based Disinfectants

Quaternary ammonium compounds (QACs) are cationic surfactants that are frequently used as disinfectants in food processing facilities and clinical environments to control bacterial growth (Bloomfield, 2002; Tezel, Pierson, and Paylostathis, 2007). Studies have confirmed the effectiveness of QACs as antiseptics against *E. coli* O157:H7, though the type of QAC may affect the efficacy of the disinfectant. *Escherichia coli* O157:H7 strains were shown to have a minimal inhibitory concentration $(MIC)_{50}$ of 2 µg/mL and an MIC_{90} of 4 µg/mL when treated with the QAC dodecyldimethyl ammonium chloride, an MIC_{50} of 16 µg/mL and an MIC_{90} of 16 µg/mL when treated with benzyldimethyldodecylammonium chloride, an MIC_{50} of 8 µg/mL and an MIC_{90} of 16 µg/mL when treated with benzyldimethyltetradecylammonium chloride, and an MIC_{50} of 8 µg/mL and an MIC_{90} of 16 µg/mL when treated with benzyldimethylhexadecylammonium chloride (Beier *et al*., 2012).

In another study, the potency of the commercial QAC-based disinfectant Vanquish (alkylbenayldimethyl-ammonium chloride) was assessed on *E. coli* O157:H7 strains acquired from beef trim enrichment samples during high-event periods (HEP), which refers to a time period in which commercial meat plants experience a higher than usual rate of *E. coli* O157:H7 contamination (Wang *et al*., 2016; Wang *et al*., 2014). Both HEP and control strains showed MICs between 0.75 and 6 ppm, though the control strains were significantly more susceptible to Vanquish than the HEP strains. Of the HEP strains, 87% showed an MIC of 6ppm, while only 32% of the control strains had MIC levels this high (Wang *et al*., 2016).

The potency of QACs on *E. coli* O157:H7 on the surface of food products has also been evaluated. *E. coli* O157:H7 in liquid suspension and inoculated on the surfaces of cantaloupes and watermelons melons were exposed to a 300 ppm solution of quaternary ammonium. It was determined that the QAC caused a mean log reduction of 6.1 ± 0.7 $\log_{10}$ (CFU/mL) for *E. coli* O157:H7 in cell suspension and a mean log reduction of 2.4 ± 0.5 $\log_{10}$ (CFU/mL) on the melon surfaces. (Svoboda *et al*., 2016). Additionally, the effectiveness of aqueous quaternary ammonium disinfectant (aQUAT, 0.02% QAC) versus an alcohol-based quaternary ammonium disinfectant (ipQUAT, 58.6% isopropyl alcohol and 0.015% QAC) at reducing *E. coli* O157:H7 on peanut and pistachio shells has been assessed (McEgan and Danyluk, 2014). The *E. coli* O157:H7 population on the peanut shells and pistachio shells was not significantly different immediately after addition of the disinfectants, but the concentration of *E. coli* O157:H7 was higher on pistachio shells (McEgan and Danyluk, 2014). After 48 hours of exposure, *E. coli* O157:H7 populations increased to 9.0 ± 0.4 $\log_{10}$ (CFU/g) on peanut shells treated with aQUAT, and populations increased to 5.5 ± 0.9 $\log_{10}$ (CFU/g) on pistachio shells. Alternatively, when shells were treated with ipQUAT, *E. coli*

O157:H7 populations declined to approximately 2 log CFU/g on peanut shells, and to concentrations below the limit of detection on pistachio shells (McEgan and Danyluk, 2014).

These studies show that *E. coli* O157:H7 is susceptible to QAC-based disinfectants, though some QACs are more effective at reducing *E. coli* O157:H7 than others. Factors in the environment such as organic matter in the surroundings may affect the usefulness of the QAC at disinfection disinfectant. Also, other chemicals such as isopropyl alcohol may be added to QAC-based disinfectants to increase their efficacy.

1.3.2 Chlorine-Based Disinfectants

Chlorine is commonly used in the produce industry as a disinfectant in washes and sprays (Cherry, 1999; Walker and LaGrange, 1991). Chlorine-based disinfectants, available in solid, liquid, or gas-injection forms, are often preferred due to their relative low cost and minimal impact on product quality (Herdt and Feng, 2009; Lou *et al*., 2012). Chlorine disinfectants actively kill a wide variety of bacteria due to their ability to oxidize thiol groups and denature proteins (Wang *et al*., 2012). Concentrations of chlorine in commonly used disinfectants can vary from 50 to 200 ppm with contact times of up to two minutes (Cherry, 1999; Walker and LaGrange, 1991). However, the presence of organic matter, high temperatures, and exposure to light can cause chlorine to rapidly lose biocidal potential (Connell and Cornell, 1996; Rodgers, 2003). These factors must be taken into account when using a chlorine-based disinfectant. Considering that hypochlorous acid, the active component of most chlorine-based disinfectants, is most abundant at pH 6.5 to 7.0, acidified chlorine compounds may also provide greater biocidal activity against *E. coli* O157:H7 and other potential pathogens (Lukasik *et al*., 2003; Herdt and Feng, 2009).

The commercial disinfectant Pro-Oxine, which contains chlorine dioxide as its active ingredient, is a commonly used disinfectant against *E. coli* O157:H7 and other potentially pathogenic bacteria (Wang *et al*., 2016). Wang *et al.* (2016) compared the resistance of 45 HEP and 47 control *E. coli* O157:H7 strains to Pro-Oxine, and MICs ranged from 1.5 to 50 ppm. High event period strains had considerably higher MICs than control strains, indicating that strains isolated during these periods in slaughter houses may be more resistant to chlorine dioxide than non-HEP strains (Wang *et al*., 2016).

Sodium hypochlorite (bleach) is commonly used as a disinfectant against a wide variety of pathogenic bacteria, among other organisms. When four *E.* coli O157:H7 strains were exposed to sodium hypochlorite solutions at various concentrations, all strains were reduced to levels below the detection limit (1.0 log CFU/mL) when free chlorine concentrations equaled or exceeded 0.5 mg/liter and exposure time equaled or exceeded 30 seconds (Shen *et al*., 2012). In another study, *E. coli* O157:H7 EDL933 from ground beef was exposed to a 0.05% sodium hypochlorite solution for 30 minutes. After incubation, there was a 6.2±0.9 log CFU/mL reduction in *E. coli* O157:H7 concentration (Sagripanti *et al*., 1997). Compared to other bacteria assayed in this study, *P. aeruginosa*, *S. Typhimurium,* and *S. aureus* had lower reductions in the same sodium hypochlorite solution, while *L. monocutogenes, S. sonnei, S. epidermidis, V. cholera, V. parahaemolyticus, V. vulnificus,* and *Y. enterocolitica* were more susceptible (Sagripanti *et al*., 1997).

An increase in organic load has been shown to significantly affect pathogen reduction in sodium hypochlorite solutions (Shen *et al*., 2012). When tomato extract was added to an 8.0 mg/liter solution of sodium hypochlorite solution, the concentration of free chlorine reduced to 0.09 mg/liter (Shen *et al*., 2012). A significantly higher initial free chlorine

concentration and a longer contact time was needed to inactivate *E. coli* O157:H7 in a 0.1% solution of lettuce extract (Shen *et al*., 2012). Similarly, in a study conducted by Williams *et al.* (2007), the researchers showed that when bleach is used to clean floor tiles contaminated with *E. coli* O157:H7, tiles that have organic matter (saw dust) on them are more likely to retain greater numbers of *E. coli* O157:H7 after disinfection than when bleach and water are used alone on tiles without organic contamination.

Chlorine-based solutions have been shown to be efficacious as a disinfectant on surfaces of some types of produce, while less efficacy is achieved on others. A previous study reported that a free chlorine solution at 50 ppm was capable of reducing *E. coli* O157:H7 loads on strawberry surfaces by 95.7% after a two minute exposure, compared to a 62.1% reduction in tap water with only 0.3 ppm of free chlorine (Lukasik *et al.*, 2002). Svoboda *et al.* (2016) inoculated the surface of melons with *E. coli* O157:H7, then exposed the melons to a 200 ppm solution of commercial bleach for two minutes. When *E. coli* O157:H7 were initially inoculated on the melons, the initial bacterial concentration was 4.2±0.2 $\log_{10}$ (CFU/g). After exposure to the chlorine solution, there was a mean log reduction of only 0.6±0.5 $\log_{10}$ (CFU/g) (Svoboda *et al*., 2016). Additionally, when Davidson *et al*. (2013) exposed iceberg lettuce that was inoculated with 5.93 $\log_{10}$ (CFU/g) of *E. coli* O157:H7 to a 30 ppm solution of free chlorine for 90 seconds, the *E. coli* O157:H7 concentration was reduced by only 0.77 $\log_{10}$ (CFU/g). This treatment of melons and iceberg lettuce with chlorine solutions does not seem to be an adequate pathogen reduction strategy considering the low level of reduction in *E. coli* O157:H7 concentration. However, Gonzalez *et al.* (2004) found that a two minute submersion in a 200 ppm sodium hypochlorite solution reduced an initial *E. coli* O157:H7 inoculum of 5.25 log CFU/g on carrots by 3.5-4.0 $\log_{10}$

(CFU/g), suggesting that chlorine solutions may be effective at reducing *E. coli* O157:H7 on some, but not all, produce surfaces.

Free chlorine often reacts with organic material, causing a reducing in its efficacy as a disinfectant; however, acidifying chlorine-based disinfectants may increase the biocidal activity of the disinfectant against *E. coli* O157:H7 on organic surfaces (Sun *et al*., 2012). In addition to the 200 ppm chlorine solution, Gonzalez *et al*. (2004) also exposed carrots inoculated with *E. coli* O157:H7 to a 1000 ppm acidified sodium hypochlorite solution in either fresh tap water or processing water, which contained an abundance of organic matter. The organic load in the processing water significantly affected the efficacy of the chlorine solution, as the chlorine solution alone was not able to inhibit growth of *E. coli* O157:H7; however, the acidified sodium chlorite solution was able to reduce the concentration of *E. coli* O157:H7 by up to 5.25 $\log_{10}$ (CFU/g) (Gonzalez *et al.*, 2004).

Chlorine solutions may also be used in animal drinking water on farms to prevent the growth of *E. coli* O157:H7; however, the presence of feces and/or rumen content in the water may decrease its efficacy (Zhao *et al*., 2006). Zhao *et al*. (2006) demonstrated that a 5 ppm chlorine solution reduced *E. coli* O157:H7 concentrations by less than 1 $\log_{10}$ (CFU/mL). Additionally, hard water, which is commonly used on farms, has been demonstrated to reduce the efficacy of chlorine as a disinfectant against *E. coli* O157:H7 when compared to deionized water (Swanson and Fu, 2017). These results indicate that chlorine in animal drinking water may not be an efficacious strategy to reduce the spread of *E. coli* O157:H7

These studies indicate that chlorine-based disinfectant solutions are capable of killing *E. coli* O157:H7 in solution as well as on the surfaces of produce, though the extent of the killing is likely dependent on the concentration of free chlorine, the exposure time, the

surface, and the abundance of organic matter.. Free chlorine solutions were demonstrated to be much more effective at reducing *E. coli* O157:H7 concentrations on some sources of produce than others. By acidifying a chlorine solution, its efficacy may be increased in the presence of an abundance of organic matter. Additionally, chlorinated water likely does not serve as an efficacious treatment for the control of *E. coli* O157:H7 in livestock drinking water, especially those with organic contamination.

1.3.3 Hydrogen Peroxide-Based Disinfectants

Hydrogen peroxide is a relatively non-toxic disinfectant that can be used to disinfect a variety of surfaces. Reactive oxygen species such as hydrogen peroxide work by oxidizing lipids, proteins, and nucleic acids, though studies suggest that the killing of *E. coli* with hydrogen peroxide is mainly due to DNA damage (Demple, 1991; Hagensee, 1989; Imlay and Linn, 1988). Treatments with hydrogen peroxide have been shown to reduce pathogen levels on produce surfaces by 1 to 6 $\log_{10}$ CFU/cm^2 (Alvarado-Casillas *et al*., 2007; Fouladkhah and Avens, 2010; Materon, 2003; Sapers *et al*., 2001).

In a study by Svoboda *et al*. (2016), a 5% hydrogen peroxide solution reduced the concentration of *E. coli* O157:H7 in suspension by 8.0±0.8 $\log_{10}$ (CFU/mL) after five minutes of exposure, and in a 35% hydrogen peroxide solution, the *E. coli* O157:H7 concentration was reduced by 9.5±0.1 $\log_{10}$ (CFU/mL) after five minutes of exposure. Additionally, Sagripanti *et al.* (1997) showed that a 10% hydrogen peroxide reduced the concentration of *E. coli* O157:H7 by greater than 6.9 log CFU/mL after 30 minutes of exposure.

Hydrogen peroxide can be used to disinfect the surfaces of a wide variety of fruits and vegetables. Lukasik *et al*. (2002) used a 0.5% solution of hydrogen peroxide to wash strawberries inoculated with *E. coli* O157:H7. They reported that this treatment reduced the concentration of *E. coli* O157:H7 on the surface of the strawberries by 97.6% (Lukasik *et al*., 2002). However, a 5% hydrogen peroxide solution used to wash *E. coli* O157:H7-inoculated melons reduced the concentration of the pathogen on the surface of the melons by only 1.4±0.5 $\log_{10}$ (CFU/mL) (Svoboda *et al*., 2016). Mei *et al*. (2017) exposed lettuce inoculated with 8 $\log_{10}$ (CFU/g) of *E. coli* O157:H7 to a 50 mM solution of hydrogen peroxide. After a 40 minute exposure, the concentration of the *E. coli* O157:H7 was reduced by 2.42±0.15 $\log_{10}$ (CFU/g) (Mei *et al*., 2017). These studies show that hydrogen peroxide solutions may be efficacious at reducing the concentration of *E. coli* O157:H7 on the surface of some types of produce, but not all.

To determine how hydrogen peroxide affected transcription of key virulence- and stress-related genes in *E. coli* O157:H7, Mei *et al.* (2017) extracted RNA from the *E. coli* O157:H7 after treatment with the 50 mM hydrogen peroxide solution. The researchers determined that genes associated with oxidative stress (*oxyR* and *sodA*), universal stress (*uspA* and *rpoS*), starvation (*phoA*), acid stress (*gadB* and *gadW*), and virulence (*stxA, stx2A,* and *fliC*) were all significantly downregulated after exposure to hydrogen peroxide (Mei *et al*., 2017). Genes related to starvation (*dps*), cold shock (*cspA*), and mismatch repair (*mutS*) were all upregulated (Mei *et al*., 2017). Two genes related to cold shock (*cspC* and *cspE*) were also upregulated, possibly due to their ability to repair damaged DNA (Mei *et al*., 2017).

Hydrogen peroxide is effective at reducing *E. coli* O157:H7 concentrations in a variety of situations, though differences in concentration and environmental conditions can produce variable results. The fact that hydrogen peroxide is not as susceptible to organic matter as chlorine make hydrogen peroxide preferable to chlorine depending upon the conditions and application of the disinfectant. However, hydrogen peroxide is not as stable as chlorine and other disinfectants when exposed to light or high temperatures. These factors should be taken into consideration when choosing an appropriate disinfectant for the elimination of *E. coli* O157:H7.

1.3.4 Other Disinfectants

A variety of other disinfectants have been shown to reduce *E. coli* O157:H7 concentrations. Many of these may be more efficacious than commonly used disinfectants. For instance, chlorine dioxide gas has been shown to be quite efficacious at inactivating *E. coli* O157:H7 and other microorganisms on fresh produce (Han *et al*., 2001). *Escherichia coli* O157:H7 concentration reductions of 3 to 4 $\log_{10}$ (CFU/ml) have been reported with chlorine dioxide at 3 to 10 ppm in water (Han *et al*., 2001; Singh *et al*., 2002). Unlike free chlorine, chlorine dioxide does not react with ammonia to form chloramines, and is thus less reactive toward organic compounds (Richardson, 1998), increasing its efficacy against *E. coli* O157:H7 and other pathogens in organically contaminated samples. On produce, a 3 ppm chlorine dioxide solution reduced *E. coli* O157:H7 by about 4.9 $\log_{10}$ (CFU/g) (Silagyi *et al*., 2009).

Peroxyacetic acid, approved by the FDA in 1986 as a food-grade disinfectant at concentrations not exceeding 100 ppm, is a noncorrosive, temperature stable alternative

disinfectant that is not affected by the presence of organic matter (Rodgers, Siddiq, and Ryser, 2003). A liquid *E. coli* O157:H7 culture of approximately 6 $\log_{10}$ (CFU/mL) was exposed to an 80 ppm peroxyacetic acid solution, and the concentration of *E. coli* O157:H7 was reduced by greater than 5 $\log_{10}$ (CFU/mL) after 70 to 75 seconds of exposure, suggesting that peroxyacetic acid may be a useful alternative to common disinfectants. However, a peroxyacetic acid disinfectant solution of the same concentration achieved only a 1.75 $\log_{10}$ (CFU/g) reduction of *E. coli* O157:H7 concentration on shredded carrots (Gonzalez *et al*., 2004), which may suggest that peroxyacetic acid may perform well as a disinfectant in an aqueous environment, but not as well on the surface of produce.

Ozonated water has been shown to be efficacious at reducing microbial populations liquid culture and on fresh produce, with reported reductions on lettuce of up to 4.6 $\log_{10}$ (CFU/g) after a five minute exposure to 1.3 mM ozone (Kim, Yousef, and Chism, 1999). Ozone is a noncorrosive, pH independent disinfectant alternative that is able to eliminate pesticides and chemical residues, as well as convert non-biodegradable organic materials into biodegradable forms (Rodgers *et al*., 2003; Graham, 1997; Hwang, 2001). A 3 ppm ozonated water solution was capable of reducing an *E. coli* O157:H7 liquid culture by greater than 5 $\log_{10}$ (CFU/mL) in as little as 15 seconds (Rodgers *et al*., 2003).

Sagripanti *et al*. (1997) compared the efficacy of a variety of disinfectants to eliminate several bacterial species including *E. coli* O157:H7. These disinfectants included a 2% solution of glutaraldehyde, 8% solution of formaldehyde, 0.03% solution of peracetic acid, 5% solution of phenol, and a cupric ascorbate solution that consisted of 0.5% cupric ions, 0.1% ascorbic acid, and 0.003% hydrogen peroxide. For *E. coli* O157:H7, the glutaraldehyde, formaldehyde, phenol, and peracetic acid solutions all reduced the

concentration of the pathogen by greater than 6.9 $\log_{10}$ (CFU/mL), which was beyond the limit of detection. Additionally, the cupric ascorbate solution reduced the *E. coli* O157:H7 concentration by 6.3±0.8 $\log_{10}$ (CFU/mL). Compared to the other bacteria assayed in this study, *E. coli* O157:H7 ranked as medium in terms of relative resistance to the variety of disinfectants that were assayed (Sagripanti *et al*., 1997).

Additionally, Zhao *et al*. (2017) demonstrated that a treatment combination of 0.1% lactic acid, 0.9% acidic calcium sulfate, and 0.05% caprylic acid added to rumen content-contaminated drinking water for cattle reduced *E. coli* O157:H7 concentrations starting at approximately 6 $\log_{10}$ (CFU/mL) by greater than 3 $\log_{10}$ (CFU/mL). This study demonstrates an efficacious disinfectant solution that may be added to farm animal drinking water to prevent the growth of *E. coli* O157:H7.

There are a variety of disinfectants that can be used to reduce concentrations of *E. coli* O157:H7, but their efficacy may vary depending on concentration and location of the application,. Several factors should be taken into account when deciding which disinfectant is best to use, such as the environmental conditions, presence of organic matter, and required concentration of the disinfectant. Though QAC, chlorine, and hydrogen peroxide are some of the most commonly used disinfectants, several other disinfectants may be efficacious at decreasing pathogen populations. While most current studies have focused on identifying the efficacy of disinfectants at killing *E. coli* O157:H7 in suspension and on produce surfaces, few studies have identified the efficacy of disinfectants at killing *E. coli* O157:H7 on farms, where direct transmission from animal to human may occur. Further work is needed to understand the advantages and disadvantages of disinfectant products against *E. coli* O157:H7 and to determine which disinfectants are most preferable for reduction of *E. coli*

O157:H7 on farms and in livestock habitats to prevent the transmission of *E. coli* O157:H7 to employees and visitors.

1.4 Susceptibility of *E. coli* O157:H7 in Biofilms to Disinfectants

Multiple studies have demonstrated that *E. coli* O157:H7 is capable of attaching to, colonizing, and forming biofilms on a variety of surfaces such as polystyrene, steel, and produce (Marouani-Gadri, Augier, and Carpenter, 2009; Silagyi, 2009; Torres *et al*., 2005; Wang *et al*., 2016). When attached to a surface in a biofilm, *E. coli* O157:H7 may occasionally detach and lead to contamination of food products or spread to a host (Wang *et al*., 2012; Silagyi *et al*., 2009). This presents a significant problem for the food processing and agritourism industry. Sanitization of food and animal contact surfaces and food products is important to prevent cross-contamination and risk of infection; however, bacteria in biofilms are usually much more tolerant to disinfectants than free-flowing planktonic cells due to protection by the ECM (Wang *et al*., 2012). Wang *et al.* (2012) demonstrated that STEC biofilm tolerance to sanitization is highly strain-dependent, regardless of serotype. Some strains of *E. coli* O157:H7 may be more resistant to disinfectants than others, leading to the possibility of sudden outbreaks (Wang *et al*., 2012). Other studies have demonstrated that concentrations of planktonic cells are generally reduced much more when exposed to disinfectant compounds than cells embedded in biofilms (Wong, 1998). Additionally, *E. coli* O157:H7 strains with stronger biofilm-forming abilities have been shown to exhibit increased resistance to disinfectants (Wang *et al*., 2014). Several different disinfectants may be used against *E. coli* O157:H7, but their efficacy at killing *E. coli* O157:H7 in a biofilm differs.

1.4.1 Quaternary Ammonium-Based Disinfectants

QAC-based disinfectants have been demonstrated to show efficacy at killing *E. coli* O157:H7 in biofilms. Fouladkhah *et al*. (2013) exposed *E. coli* O157:H7 biofilms on stainless steel to a 240 ppm solution of the QAC-based disinfectant Oasis-146 Multi-Quat for one minute, then allowed the biofilm to recover. Seven days post-treatment, cells in the biofilm were enumerated, and concentrations were reduced from 2.4 ± 0.6 $\log_{10}$ (CFU/cm^2) to less than 0.7 ± 0.6 $\log_{10}$ (CFU/cm^2) (Fouladkhah *et al*., 2013). Treatment with water alone produced concentrations of 1.2 ± 0.8 to 1.9 ± 0.8 $\log_{10}$ (CFU/cm^2) (Fouladkhah *et al*., 2013).

The QAC-based disinfectant Vanquish has been assayed for its efficacy against *E. coli* O157:H7 in biofilms (Wang *et al*., 2012). Three strains of *E. coli* O157:H7 were induced to form biofilms on 96-well polystyrene plates, and the biofilms were exposed to a 300 ppm solution of the disinfectant. After two minutes of exposure to the disinfectant solution, biofilm cell counts were between 4.78 and 5.89 $\log_{10}$ (CFU per well), compared to 7.28 to 7.50 $\log_{10}$ (CFU per well) in the untreated controls (Wang *et al*., 2012). After a ten minute exposure, viable cell counts of the treated biofilms ranged from 1.02 to 4.37 $\log_{10}$ (CFU per well), while cell counts in the untreated biofilms ranged from 7.35 to 7.47 $\log_{10}$ (CFU per well) (Wang *et al*., 2012). This demonstrates that there was a reduction in the number of viable cells within the biofilms after exposure to the disinfectant Vanquish, but biofilm tolerance was strain-dependent as the concentration of one strain of *E. coli* O157:H7 in biofilms was not reduced as much as the other two strains after 10 minutes of exposure to the disinfectant (4.37 $\log_{10}$ (CFU per well) compared to 1.02 and 1.47 $\log_{10}$ (CFU per well); Wang *et al*., 2012).

The same QAC-based disinfectant Vanquish was assayed against HEP strains of *E. coli* O157:H7 in biofilm and compared to *E. coli* O157:H7 controls strains (Wang *et al*., 2014). The researchers suspected that the high event period of *E. coli* O157:H7 contamination could have been due increased resistance to the disinfectants in the HEP strains. A 300 ppm quaternary ammonium solution of Vanquish was used to treat HEP and control strains in biofilms for one minute, and the biofilms were neutralized and allowed to recover. Of the HEP strains, 36% were moderate survivors and 64% were weak survivors (Wang *et al*., 2014). In comparison, only 12.8% of the control strains were moderate survivors and 55.3% were weak survivors (Wang *et al*., 2014). Overall, the HEP strains were more tolerant to the quaternary ammonium disinfectants than the control strains, indicating that the high event period may have been due to increased disinfectant tolerant.

Additionally, the efficacy of Vanquish has been assayed on dual-strain biofilms containing *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium (Wang *et al*., 2013). The dual-strain biofilms were exposed to the 300 ppm quaternary ammonium solution for one minute. The researchers demonstrated that *E. coli* or *Salmonella* strains that were unable to produce extracellular polymeric substances (EPS) on their own had significantly enhanced survival after exposure to the disinfectant when they were grown in biofilms with the other species that could produce EPS (Wang *et al*., 2014).

These studies demonstrate that QAC-based disinfectnats may be able to reduce *E. coli* O157:H7 concentrations in biofilms, but these reductions are not as significant as reductions in concentrations of planktonic cells. The biofilms likely provide protection to the embedded cells to shield them from the disinfectant compound. The current studies point towards *E. coli* O157:H7 in biofilms as more resistant or tolerant to QAC-based disinfectants than

planktonic cells, but more work is needed to determine if the cells are truly resistant in a biofilm state and continue to grow within the biofilm during exposure to disinfectants, or if the biofilm merely protects them and provides tolerance to the disinfectants.

1.4.2 Chlorine-Based Disinfectants

Chlorine can be used to depolymerize and remove the exopolysaccharide matrix that surrounds and protects bacteria in biofilms, making it a candidate for an efficacious disinfectant to eliminate *E. coli* O157:H7 in biofilms (Wang *et al*., 2012). Wang *et al.* (2012) exposed *E. coli* O157:H7 in biofilms within a 96-well polystyrene plate to a 200 ppm sodium hypochlorite solution for 2 to 10 minutes, and cells in the biofilms were enumerated. After two minutes of exposure, cell counts ranged from 3.16 to 5.31 $\log_{10}$ (CFU per well), while after 10 minutes, cell counts ranged from 0.71 to 4.42 $\log_{10}$ (CFU per well). Untreated biofilms contained 7.35 to 7.47 (CFU per well) (Wang *et al*., 2012). Compared to the QAC-based disinfectant mentioned previously in this study, the concentration of *E. coli* O157:H7 in biofilms were generally reduced in greater amounts when the chlorine-based disinfectant was used (Wang *et al*., 2012).

Wang *et al*. (2014) also determined the efficacy of chlorine-based disinfectants at killing HEP strains of *E. coli* O157:H7 in biofilms. As mentioned previously, mature HEP strain biofilms were exposed to the disinfectant solution, this time a 200 ppm solution of sodium hypochlorite, for one minute, neutralized, and then allowed 18 to 20 hours to recover (Wang *et al*., 2014). Of the HEP strains, 36% and 64% were moderate and weak survivors, respectively. These results were compared with control strains, which showed 13% as moderate survivors and 55% as weak survivors (Wang *et al*., 2014). Interestingly, these

results were the same as when the QAC-based disinfectant was used against HEP and control strains, suggesting that the chlorine and quaternary ammonium disinfectants used in this study are equally effective against *E. coli* O157:H7 in biofilm (Wang *et al*., 2014).

Studies have also determined the efficacy of chlorine-based disinfectants against *E. coli* O157:H7 biofilms that have formed on stainless steel (Sharma, Ryu, and Beuchat, 2005; Bae, Baek, and Lee, 2012). Sharma *et al*. (2005) grew *E. coli* O157:H7 biofilms on stainless steel coupons, then exposed the coupons to chlorine solutions of 50 and 100 µg/mL. The *E. coli* O157:H7 concentrations were significantly reduced from 8 $\log_{10}$ (CFU per coupon) when treated with water alone to around 3.5 $\log_{10}$ (CFU per coupon) after treatment with the chlorine solution (Sharma, Ryu, and Beuchat, 2005). No significant difference was noticed between the 50 and 100 µg/mL chlorine solutions (Sharma, Ryu, and Beuchat, 2005). Bae *et al*. (2012) also grew *E. coli* O157:H7 biofilms on stainless steel coupons and exposed the biofilms to Clean Shot, a chlorine-based disinfectant, for one to five minutes. Before treatment, there were approximately 5 to 7 $\log_{10}$ (CFU per coupon) (Bae, Baek, and Lee, 2012). After treatment with only distilled water, populations were slightly reduced to 4 to 6 $\log_{10}$ (CFU per coupon) after one minute and 3 to 5 $\log_{10}$ (CFU per coupon) after five minutes (Bae, Baek, and Lee, 2012). Treatment with the chlorine disinfectant reduced the *E. coli* O157:H7 populations to 2.6 to 4 $\log_{10}$ (CFU per coupon) after one minute and to less than 1.5 to 1.7 $\log_{10}$ (CFU per coupon) after five minutes of exposure (Bae, Baek, and Lee, 2012). These results demonstrate that

These studies demonstrate that chlorine-based disinfectant compounds may be efficacious at reducing *E. coli* O157:H7 concentrations in biofilms, but none of the methods used in these studies achieved true disinfection as *E. coli* O157:H7 was still recovered after

application of the disinfectant. Also, free chlorine concentrations must be increased to deactivate *E. coli* O157:H7 in biofilms compared to planktonic cells considering concentrations of 200 ppm free chlorine were required to effectively reduce cell counts in biofilms, whereas MICs for chlorine dioxide ranged from 1.5 to 50 ppm according to other studies (Wang *et al*., 2012; Wang *et al*., 2016). More research is needed to directly compare similar chlorine treatments against *E. coli* O157:H7 in planktonic and biofilm forms. Also, since it has been demonstrated that acidified chlorine solutions increased its efficacy against planktonic *E. coli* O157:H7 (Gonzalez *et al*., 2004), similar studies should be conducted to identify the efficacy of acidified chlorine solutions against *E. coli* O157:H7 in biofilms.

1.4.3 Other Disinfectants

Other disinfectant compounds may be used to reduce *E. coli* O157:H7 concentrations in biofilms, though their efficacy is variable. Fouladkhah *et al*. (2013) used an acid-based disinfectant consisting of 6.9% hydrogen peroxide, 4.4% peroxy acetic acid, and 3.3% octanoic acid to treat mature *E. coli* O157:H7 biofilms on stainless steel, and the biofilms were allowed to recover after treatment. Seven days post treatment, biofilm cells were reduced from 2 $\log_{10}$ (CFU/cm^2) to 1.2 ± 0.5 $\log_{10}$ (CFU/cm^2) (Fouladkhah, Geornaras, and Sofos, 2013). This treatment was not as effective as quaternary ammonium, which reduced the concentration of *E. coli* O157:H7 in the biofilm to less than 0.7 ± 0.6 $\log_{10}$ (CFU/cm^2) in the same study (Fouladkhah, Geornaras, and Sofos, 2013).

Bae *et al*. (2012) exposed *E. coli* O157:H7 biofilms on stainless steel to an alcohol-based disinfectant. After one minute and five minute treatments, *E. coli* O157:H7 concentrations in the biofilms were reduced from 3 to 5 $\log_{10}$ (CFU per coupon) to below

1.48 $\log_{10}$ (CFU per coupon) (Bae, Baek, and Lee, 2012). These results suggest that alcohol was very effective at removing *E. coli* O157:H7 biofilms, more so than most other treatments (Bae, Baek, and Lee, 2012). Future work should further investigate the efficacy of alcohol-based disinfectants at removing *E. coli* O157:H7 biofilms.

Additionally, Kim and Rhee (2016) demonstrated that phytic acid solutions with sodium chloride are efficacious against *E. coli* O157:H7 in biofilms. *Escherichia coli* O157:H7 biofilms were exposed to a 0.4% phytic acid solution with 3-4% sodium chloride for five minutes. The disinfectant solution completely inactivated *E. coli* O157:H7 within the biofilm (Kim and Rhee, 2016). Beyond this study, little research has been conducted on the anti-biofilm effects of phytic acid with sodium chloride, but more work should be done to confirm these incidations of phytic acid with sodium chloride as a highly efficacious treatment to inactivate *E. coli* O157:H7 in biofilms.

Relatively few studies are available that have determined the efficacy of disinfectant compounds against *E. coli* O157:H7 in biofilms. Since commonly used disinfectants tend to require higher concentrations to adequately reduce *E. coli* O157:H7 concentrations in biofilm versus in a planktonic form, future research should investigate the efficacy of alternative products to remove *E. coli* O157:H7 biofilms and kill the bacteria living in them. Alcohol and phytic acid with sodium chloride solutions seem promising as anti-biofilm disinfectants (Bae, Baek, and Lee, 2012; Kim and Rhee, 2016), and other disinfectant products should be evaluated for their anti-biofilm effects.

1.5 Conclusions

Since *E. coli* O157:H7 can cause severe gastrointestinal and systemic diseases, control of the pathogen should be optimized through adequate disinfection strategies. The susceptibility of *E. coli* O157:H7 to disinfectants may be reduced through the formation of biofilms. Many studies in the current body of literature have thoroughly described the process of biofilm formation by *E. coli* O157:H7, and recent studies have demonstrated the increased tolerance of *E. coli* O157:H7 in biofilms to commonly used disinfectants as compared to planktonic forms of *E. coli* O157:H7. While commonly used disinfectants such as quaternary ammonium compounds and chlorine-based compounds appear to significantly reduce *E. coli* O157:H7 concentrations in suspension, they are less efficacious at reducing *E. coli* O157:H7 concentrations in biofilms. Further work is needed on a variety of disinfectants, including less commonly used disinfectants, to identify the most appropriate and efficacious disinfectants at killing *E. coli* O157:H7 in planktonic form as well as in biofilms. Additionally, few studies have determined the efficacy of disinfectants at killing *E. coli* O157:H7 in farming or livestock-interaction settings. Further work is needed to identify and evaluate the most efficacious disinfectants to use in these settings to reduce concentrations of *E. coli* O157:H7 to prevent future transmission of the pathogen.

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CHAPTER 2

Biofilm-forming potential of *Escherichia coli* O157:H7 on plastic and rubber footbath surfaces

Abstract

Escherichia coli O157:H7 is a human pathogen that can cause severe gastrointestinal disease. Outbreaks have occurred at livestock interaction events and have been associated with asymptomatic ruminants. Footbath use is a pathogen-control strategy that may be used at agritourism events to reduce infection risk. The potential of *E. coli* O157:H7 to form biofilms on environmental surfaces, including footbaths, is unknown. Our objective was to determine the biofilm-forming potential of *E. coli* O157:H7 on polystyrene plastic and rubber footbath surfaces. Genetically distinct *E. coli* O157:H7 strains were evaluated for their biofilm-forming potential on polystyrene pegs incubated for 24, 48, and 72 hours at 22˚C, 37˚C, and 42˚C, and on rubber footbath pieces incubated for 24 hours at 22˚C, 37˚C, and 42˚C. Crystal violet staining and biofilm CFU assays were performed to determine biofilm biomass and $\log_{10}$(CFU/mL) values. Variations in biofilm density were observed between strains, times, and temperatures with a significant three-way interaction ($p < 0.0001$) on polystyrene. Lower incubation temperatures and longer incubation times were associated with more dense biofilms, with no significant biofilm formation at 24 hours. On rubber footbaths, the strains produced biofilm in a strain and temperature dependent manner, with a significant two-way interaction ($p = 0.02$). The most dense biofilms were formed at 22˚C on rubber footbath surfaces, though these biofilms were not mature. This study demonstrates that *E. coli* O157:H7 biofilm formation is strain, temperature, and time dependent, and that *E. coli* O157:H7 may begin to form biofilm on rubber footbath surfaces within 24 hours.

Importance

Escherichia coli O157:H7 is an important pathogen that can cause severe gastrointestinal and systemic diseases in humans, particularly in young children. *Escherichia coli* O157:H7 can be carried asymptomatically in the intestines of ruminants and shed in their feces. The bacteria can spread to humans by ingestion of contaminated food or water, or through direct contact. Possible outbreaks of *E. coli* O157:H7 at agritourism events are of concern, and biosafety measures such as footbath use can be implemented to decrease the risk of infection. If *E. coli* O157:H7 strains are capable of forming biofilms on rubber footbath surfaces, their susceptibility to disinfectants in the footbaths could be decreased, and they could persist in the footbaths for longer periods, causing the footbaths to become a potential source of contamination. This study aims to identify what conditions *E. coli* O157:H7 optimally forms biofilms, and whether they can form biofilms on rubber footbath surfaces.

2.1 Introduction

Escherichia coli O157:H7 is a human pathogen that can cause several severe diseases such as bloody diarrhea, thrombotic thrombocytopenic purpura, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Doyle, 1991; Mead and Griffin, 1998; Wang *et al.*, 2012). Hemolytic uremic syndrome, which develops in 2 to 7% of people with *E. coli* O157:H7-associated bloody diarrhea, is a life-threatening complication that can lead to kidney failure, particularly in young children and the elderly (Durso, Bono, and Keen, 2005; Easton, 1997). From 2003 to 2012, 390 *E. coli* O157 outbreaks were reported in the United States, resulting in 4,928 illnesses, 1,272 hospitalizations, 299 diagnosed cases of HUS, and 33 deaths (Heiman *et al*., 2015).

Escherichia coli O157:H7 naturally exists in the digestive tracts of ruminants, where the organisms colonize and can be asymptomatically shed in the feces. *Escherichia coli* O157:H7 infections can be acquired from consumption of contaminated food or water products, or from direct contact and interaction with ruminants or their environment (Nguyen and Sperandio, 2012). Direct contact is of particular concern to children that are visiting petting zoos, farms, and other agritourism or livestock interaction events. Outbreaks of *E. coli* O157:H7 have been associated with agritourism events across the United States, including recently in Connecticut where six patients who visited the same goat dairy farm were diagnosed with *E. coli* O157 infections (Centers for Disease Control and Prevention, 2006; Saulo, 2013; Laughlin *et al*., 2016).

Simple biosafety measures to control pathogen transmission at livestock interaction events may include handwashing stations, separation of dining and livestock handling areas, and use of footbaths. The implementation of these measures may reduce the occurrence of

such outbreaks. Footbaths are disinfectant-filled mats or pans placed at the exits of animal habitats to clean soiled boots and shoes to prevent spreading pathogens outside of the contaminated area. Several studies have shown that footbaths may significantly decrease viable bacterial load on shoes and floors, although efficacy can be variable depending upon the type of disinfectant in the footbath (Hornig *et al.*, 2016; Allen *et al*., 2010; Dunowska *et al*., 2006; Hartmann, Dusick, and Young, 2013).

Biofilm formation by *E. coli* O157:H7 is a concern for pathogen control due to the potential for biofilms to persist within the environment and confer resistance to disinfectants (Bridier *et al*., 2011; Shi and Zhu, 2009). A biofilm is a population of bacteria of the same or different species that are enclosed in a protective extracellular matrix (ECM) and have altered gene transcription and phenotypic characteristics such as a different growth rate than planktonic cells (Saur, Rickard, and Davies, 2007; Donlan and Costerton, 2002). Biofilms offer metabolic benefits and physical protection to bacteria in harsh environments (Watnick and Kolter, 2000). Studies have shown that bacteria in biofilms are more likely than planktonic cells to survive sanitation measures due to protection by the ECM (Bridier *et al*., 2011; Mah and O'Toole, 2001; Stewart and Costerton, 2001; Chmielewski and Frank, 2003).

The defining feature that differentiates biofilm from planktonic cells is the presence of an ECM. Biofilm ECMs can be composed of extracellular proteins, polysaccharides, and/or DNA. *Escherichia coli* biofilms have been shown to be composed of proteins (curli and fimbriae) and polysaccharides (cellulose, colonic acid, and poly-N-acetyl glucosamine) (Uhlich, Rogers, and Mosier, 2010). Curli, the proteinaceous extracellular fibers commonly expressed in *E. coli* and *Salmonella* spp., are involved in surface attachment and cell-to-cell contact that promotes biofilm formation (Barnhart and Chapman, 2006). Curli fimbriae

components are encoded by the *csgBAC* and *csgDEFG* operons (Hammar *et al*., 1995; Romling, 2005). The *csgA* gene encodes CsgA, the major structural subunit of curli fimbriae, while *csgD* encodes CsgD, a positive transcriptional regulator of the *csgBAC* operon (Barnhart and Chapman, 2006). A previous study found that all *E. coli* O157 strains had the *csgA* gene, although curli expression was only observed in 28% of the strains (Biscola *et al.*, 2011). Also important to *E. coli* biofilm formation are extracellular polysaccharides such as the linear homopolymer Poly-β-1,6-*N*-Acetyl-D-Glucosamine (PGA). This polysaccharide serves as an adhesin to promote the structural stability of biofilms in many species of eubacteria (Itoh *et al*., 2008). The *pgaABCD* operon provides the gene products necessary for PGA synthesis (Itoh *et al*., 2008). PgaC is a glycosyltransferase required for PGA synthesis, while PgaA exports the polysaccharide to the ECM (Itoh *et al*., 2008; Wang *et al*., 2004) The *pgaABCD* operon has been shown to be ubiquitous in *E. coli*, and homologous loci have been identified in a variety of other bacterial pathogens (Wang *et al*., 2004; Cerca and Jefferson, 2008; Itoh *et al*., 2008) .

Previous studies have demonstrated that *E. coli* O157:H7 is capable of attaching to, colonizing, and forming biofilms on a wide variety of surfaces including plastic, stainless steel, and organic plant surfaces (Wang *et al*., 2012; Silagyi *et al*., 2009; Maxcy, 1971). Little research has been done to examine the biofilm-forming potential of *E. coli* O157:H7 on rubber, a common material used to make footbaths. If *E. coli* O157:H7 is capable of forming biofilm on rubber footbath surfaces, the susceptibility of the bacteria to disinfectants in the footbaths may be decreased, thus reducing the efficacy of the footbath. Our objective in this study was to identify the biofilm-forming potential of *E. coli* O157:H7 on polystyrene plastic and rubber footbath surfaces at various incubation times and temperatures.

2.2 Results

2.2.1 Biofilm biomass and CFU assays on polystyrene plastic

Seven *E. coli* O157:H7 strains and the biofilm-positive control strain *E. coli* ATCC 25922 formed biofilm, as measured by biomass (OD_{550}) and recovered CFUs ($\log_{10}$(CFU/mL)), in a strain, time, and temperature dependent manner on polystyrene plastic pegs, with a significant three-way interaction ($p < 0.0001$; Table A.1). The ranges of the OD_{550} and $\log_{10}$(CFU/mL) values for all strains at all conditions were divided into six categories and ranked 0-5. The values that ranked 4 or 5 were considered prominent ($OD_{550} > 0.049$; $\log_{10}$(CFU/mL) > 6.59). The majority of the strains did not form prominent biofilm in terms of biomass or recovered CFUs under most conditions (Figures 2.1A and 2.1B), while a few strains were capable of forming prominent biofilms under certain conditions.

At 24 hours of growth, no prominent biomass was formed by any strain at any temperature on polystyrene plastic (Figures 2.2A and 2.2D), and there was no significant difference in the OD_{550} values between strains at any temperature ($p > 0.05$; Table A.1). While there were significant differences between the $\log_{10}$(CFU/mL) values between strains ($p < 0.05$; Table A.1), it is not apparent that these values represent biofilm growth.

At 48 hours, a greater amount of biomass and recovered CFUs were observed across several strains (Figures 2.2B and 2.2E). The *E. coli* ATCC 25922 biofilm-positive control strain displayed prominent biomass and $\log_{10}$(CFU/mL) values at 22˚C and 37˚C, while two O157:H7 strains had prominent biomass values and one O157:H7 strain had a prominent $\log_{10}$(CFU/mL) value at 22˚C. The OD_{550} and $\log_{10}$(CFU/mL) values of the ATCC 25922 positive control strain and strain 171F were significantly different from the other strains at 22˚C ($p < 0.0001$; Table A.1).

Overall, the samples that were incubated for 72 hours had the most biomass and recovered bacterial concentration on polystyrene plastic (Figures 2.2C and 2.2F). ATCC 25922 displayed prominent OD_{550} values at all three temperatures and prominent $\log_{10}$(CFU/mL) values at 22˚C and 37˚C. At 22˚C, four O157:H7 strains displayed prominent OD_{550} and $\log_{10}$(CFU/mL) values. Two of these strains displayed prominent OD_{550} values at 37˚C, and three strains displayed prominent $\log_{10}$(CFU/mL) values at 37˚C. Only one O157:H7 strain a displayed prominent OD_{550} value at 42˚C, though this was not significantly different from the other strains at this temperature ($p > 0.05$; Table A.1).

In general, the strains that were incubated at 42˚C had the lowest OD_{550} and $\log_{10}$(CFU/mL) values, while those incubated at 22˚C and 37˚C had higher OD_{550} and $\log_{10}$(CFU/mL) values. An incubation time of 24 hours was not long enough for any strain to form biofilm on polystyrene plastic, but the density of the biofilms in terms of biomass and recovered CFUs increased at 48 and 72 hours (Figure 2.2). Certain strains were more prone to form biofilm than others. For instance, 171F formed prominent biofilm at 48 hours of incubation at 22˚C and after 72 hours at 22˚C and 37˚C, while 230C did not form biofilm under any condition. On polystyrene plastic, the amount of biomass was generally consistent with the $\log_{10}$(CFU/mL) values of each strain at each temperature, as can be seen by the consistencies between strains with both prominent biomass and prominent $\log_{10}$(CFU/mL) values (Figure 2.2).

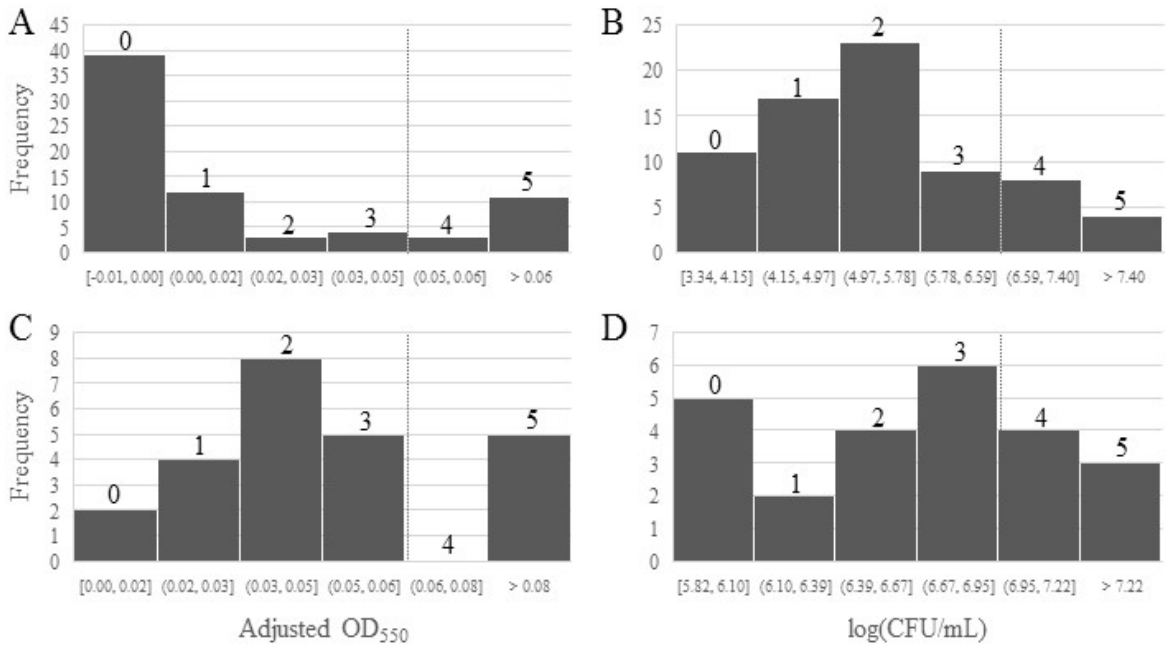

Figure 2.1. Histograms and ranking of adjusted OD_{550} and log_{10}(CFU/mL) values obtained from *Escherichia coli* biofilms grown on polystyrene plastic and rubber footbaths at various conditions. Biofilms were allowed to grow on polystyrene plastic for 24, 48, and 72 hours at 22˚C, 37˚C, and 42˚C (A and B), and on rubber footbaths for 24 hours at 22˚C, 37˚C, and 42˚C (C and D). The biomass data sets (A and C) and log_{10}(CFU/mL) data sets (B and D) were divided into six categories, ranked 0-5. Values ranked 4 or 5 (to the right of the dotted lines) were considered prominent.

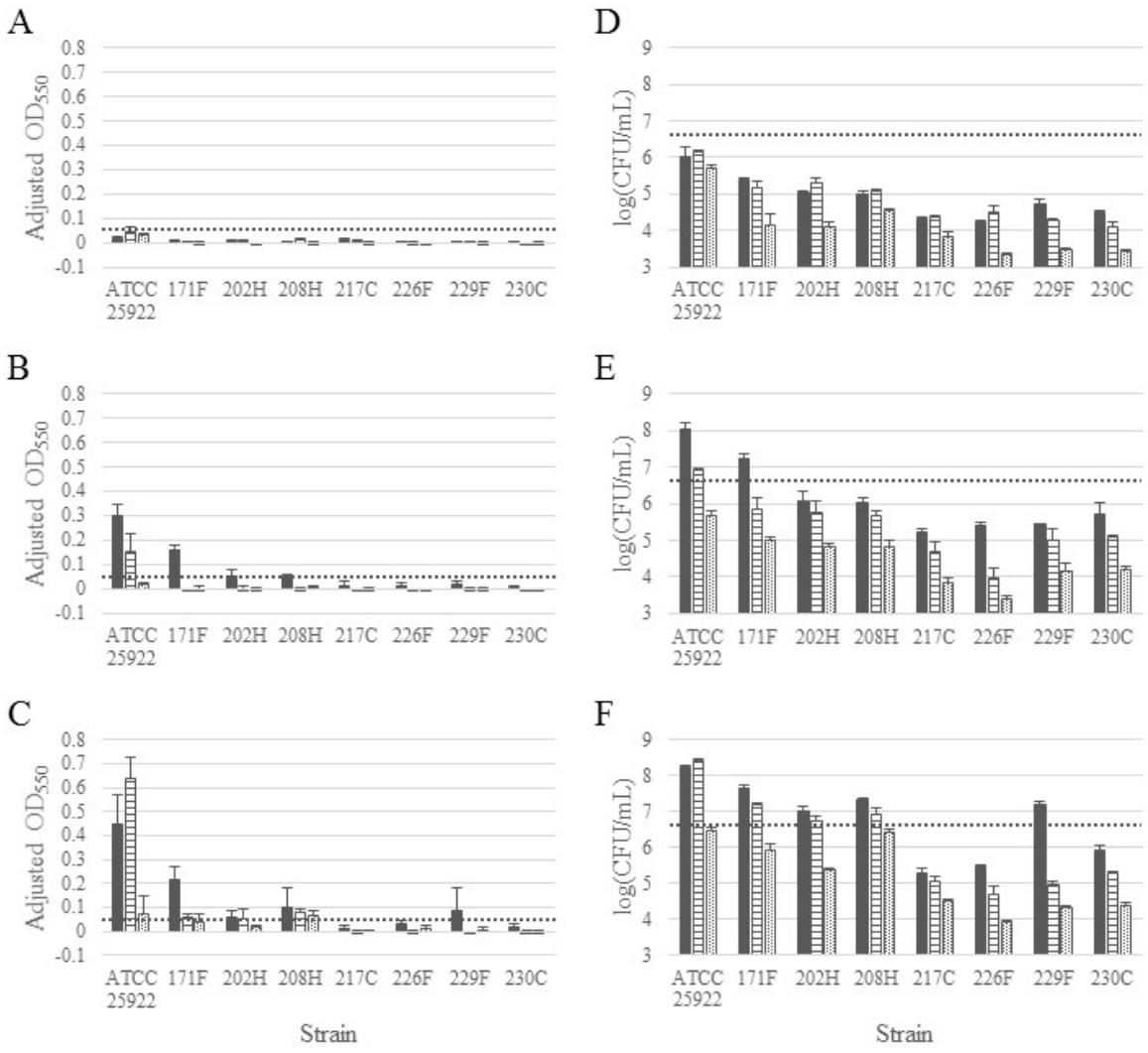

Figure 2.2. **Adjusted OD_{550} and concentration (log_{10} CFU/mL) values of *Escherichia coli* biofilms grown on polystyrene pegs.** The cultures were incubated at 22˚C (solid bars), 37˚C (bars with horizontal lines), and 42˚C (bars with dots) for 24 hours (A and D), 48 hours (B and E), and 72 hours (C and F). OD_{550} values were adjusted by subtracting the value of the uninoculated control. Dotted lines represent the values considered prominent (OD_{550} > 0.049, A-C; log_{10}(CFU/ml) > 6.59, D-F).

2.2.2 Biofilm biomass and CFU assays on rubber footbaths

Similar to the assays on polystyrene plastic, the *E. coli* O157:H7 strains formed biofilm in a strain and temperature dependent manner on the rubber footbath pieces, with a significant two-way interaction ($p < 0.0001$). Values that ranked 4 or 5 were considered

prominent for biomass (OD_{550} > 0.065) and recovered bacterial concentration (greater than 6.95 $\log_{10}$ CFU/mL) (Figures 2.1C and 2.1D).

At 22˚C, four of the O157:H7 strains had prominent biomass values (Figure 2.3A), though most of these were not significantly different than the other strains at this temperature (Table A.2). The only significantly different strains were 202H and 229F ($p = 0.0184$). Four O157:H7 strains also had prominent $\log_{10}$(CFU/mL) values at this temperature (Figure 2.3B). The strains with the highest $\log_{10}$(CFU/mL) values (230C and 217C) were significantly different from the strains with lower $\log_{10}$(CFU/mL) values ($p < 0.0005$; Table A.2), but the other two strains with prominent $\log_{10}$(CFU/mL) values at 22˚C were not significantly different from the lower-ranking strains ($p > 0.05$; Table A.2). There were no strains that formed prominent biofilms in terms of biomass or recovered bacterial concentrations at 37˚C or 42˚C on the rubber footbaths (Figure 2.3). Though the $\log_{10}$(CFU/mL) values of some strains were significantly different ($p < 0.05$) from others at 37˚C and 42˚C, none were significantly different in their OD_{550} values (Table A.2).

In general, the *E. coli* strains had higher OD_{550} and $\log_{10}$(CFU/mL) values at 22˚C than 37˚C or 42˚C (Figure 2.3). Interestingly, on rubber footbaths, the *E. coli* ATCC 25922 biofilm-positive control strain did not always have the highest OD_{550} or $\log_{10}$(CFU/mL) values. Compared to biofilms grown on polystyrene plastic, the biomass and recovered CFUs of the biofilms grown on rubber footbaths did not always correlate. For instance, strain 202H had the highest OD_{550} value of all strains at 22˚C, yet its $\log_{10}$(CFU/mL) value was not prominent.

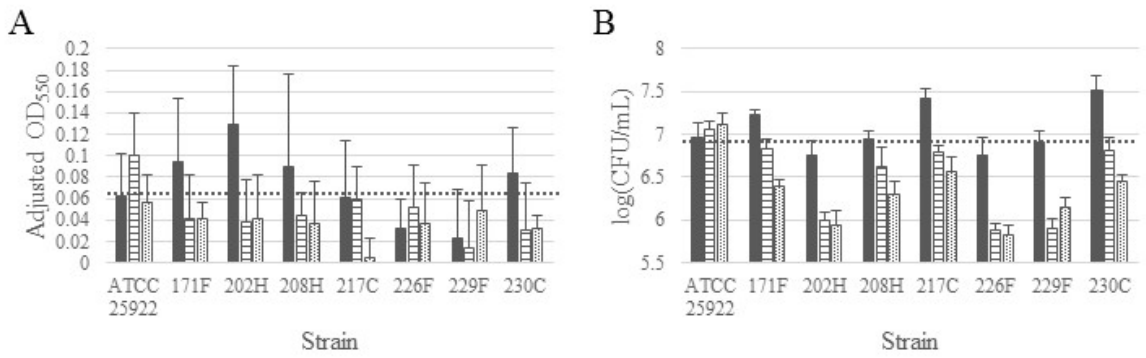

Figure 2.3. Adjusted OD_{550} and log_{10}(CFU/mL) values of *Escherichia coli* biofilms grown on rubber footbath pieces. The cultures were incubated for 24 hours at 22˚C (solid bars), 37˚C (bars with horizontal lines), and 42˚C (bars with dots).OD_{550} values were adjusted by subtracting the value of the uninoculated control. Dotted lines represent the values considered prominent (OD_{550} > 0.065, A; log_{10}(CFU/mL) > 6.95, B).

2.2.3 PCR characterization

All seven *E. coli* O157:H7 strains were positive for *fimH* (type 1 fimbriae adhesin), *csgA* and *csgD* (curli fimbriae), as well as *pgaC* and *pgaA* (adhesin polysaccharide). All strains were negative for *bfp* (bundle forming pilus) and *sfa* (S fimbrial adhesin) (Table 2.1).

Table 2.1. PCR results of biofilm-associated genes in *Escherichia coli* O157:H7 strains.

Strain	*bfp*	*fimH*	*sfa*	*csgA*	*csgD*	*pgaC*	*pgaA*
171F	-	+	-	+	+	+	+
202H	-	+	-	+	+	+	+
208H	-	+	-	+	+	+	+
217C	-	+	-	+	+	+	+
226F	-	+	-	+	+	+	+
229F	-	+	-	+	+	+	+
230C	-	+	-	+	+	+	+

2.2.4 Antimicrobial susceptibility

As shown in Table 2.2, all strains of *E. coli* O157:H7 displayed minimum inhibitory concentrations (MIC) low or below the detection limit for amoxicillin/clavulanate (4-8 µg/mL), chloramphenicol (4-8 µg/mL), ciprofloxacin (<= 0.015 µg/mL), gentamicin (0.5-1

µg/mL), and trimethoprim/sulfamethoxazole (<= 0.12 µg/mL). Six strains displayed MICs of 2-4 µg/mL towards ampicillin, while one strain (171F) displayed an MIC greater than 32 µg/mL of ampicillin; however, this was expected due to the fact that ampicillins are older antibiotics that have been used extensively.

Table 2.2. Minimum inhibitory concentration (MIC; µg/mL) of antimicrobials for seven genetically distinct *Escherichia coli* O157:H7 strains.

Strain	Amox/Clav	Ampicillin	Chloramphenicol	Ciprofloxacin	Gentamicin	Trimeth/Sulfameth
171F	8	>32	8	<=0.015	1	<=0.12
202H	4	4	8	<=0.015	0.5	<=0.12
208H	4	4	8	<=0.015	0.5	<=0.12
217C	4	2	8	<=0.015	0.5	<=0.12
226F	4	4	4	<=0.015	0.5	<=0.12
229F	4	4	4	<=0.015	0.5	<=0.12
230C	4	2	8	<=0.015	0.5	<=0.12

2.3 Discussion

Escherichia coli O157:H7 infections are of concern, particularly in young children visiting agritourism events, due to the potential severity of the diseases they cause. Ruminants at these events can asymptomatically shed the bacteria and spread the pathogen throughout the interaction environment and to visitors. Footbaths and other biosafety measures may be used to decrease the risk of infection during agritourism events; however, biofilm formation by *E. coli* O157:H7 on footbath surfaces could decrease the efficacy of footbath use.

Our results suggest that seven genetically distinct *E. coli* O157:H7 isolates, originally isolated from North Carolina meat goats, form biofilm in a strain, time, and temperature

dependent manner *in vitro* on polystyrene plastic surfaces ($p < 0.0001$). Lower temperatures (22°C) and increased incubation times (72 hours) were more favorable for prominent biofilm growth. No biofilm was formed by any strain at 24 hours, but biomass and recovered bacterial concentrations were higher for several strains at 72 hours of growth, with the most dense biofilms forming at 22˚C and less dense biofilms forming at 37˚C. *Escherichia coli* O157:H7 and other *E. coli* serotypes have been previously reported to form biofilm in a strain and temperature dependent manner on polystyrene, with greater biofilm densities produced at 25°C than 37°C (Uhlich *et al*., 2014). It has also been previously reported that *E. coli* O157:H7 and other serotypes form more dense biofilms after 72 hours of growth on polystyrene as opposed to 24 or 48 hours when incubated at 22 to 25°C (Wang *et al*., 2012).

This study uniquely assessed the ability of *E. coli* O157:H7 to form biofilm on a rubber footbath surface, which occurred as soon as 24 hours and in a strain and temperature dependent manner ($p = 0.02$). The only temperature at which prominent OD_{550} and $\log_{10}$(CFU/mL) values were observed for the O157:H7 strains was 22°C, though most of these strains were not significantly different from each other. With the exception of the biofilm-positive control strain *E. coli* ATCC 25922, strains grown at 37°C and 42°C did not produce prominent biofilms. Interestingly, ATCC 25922 formed more dense biofilms at 37˚C on rubber footbaths than at 22˚C, with several *E. coli* O157:H7 strains forming greater amounts of biomass than the control strain at 22°C. Considering the fact that little significance was observed between strains in terms of biomass values, it is likely that biofilms formed on rubber footbaths at only 24 hours are immature. The initial assays using polystyrene plastic and reports from others have shown that biofilms form in greater densities after 48 to 72 hours of incubation (Wang *et al*., 2012), so it is likely that *E. coli* O157:H7

strains would be associated with increased biofilm potential on rubber footbath surfaces if held longer. We opted to end footbath experiments at 24 hours in this study because of the likelihood that footbaths are changed daily. Also, biofilm biomass and $\log_{10}$(CFU/mL) values did not seem to closely correlate on rubber footbaths as compared to biofilms on polystyrene. For instance, strain 217C did not form significant biomass at 22˚C compared to other strains, but its $\log_{10}$(CFU/mL) value was one of the highest of the seven *E. coli* O157:H7 strains. This could be attributed to the bacteria's ability to more easily adhere to rubber as compared to polystyrene plastic surfaces. Differences in adhesion factors could attribute to the ability of the *E. coli* O157:H7 strains to adhere differently to rubber versus polystyrene, whether or not they go on to form biofilm (Garrett, Bhakoo, and Zhang, 2008). Additionally, it has previously been shown that the ability of *E. coli* O157:H7 to bind to alfalfa sprouts, human epithelial cells, and plastic is determined by a variety of surface structures expressed on the cells' outer membranes (Torres *et al*., 2005).

PCR characterization showed that all seven strains of *E. coli* O157:H7 used in this study were positive for biofilm-associated genes including *fimH, csgA, csgD, pgaC,* and *pgaA*, while none were positive for *bfp* or *sfa* genes. Despite displaying the genotypes necessary for biofilm formation, some strains did not form biofilm, while others formed biofilm only under certain conditions. For instance, strain 226F formed little to no biofilm under any conditions on either polystyrene or rubber surfaces, while 171F formed prominent biofilm on polystyrene after 48 and 72 hours of growth at 22°C, after 72 hours at 37°C, and on rubber footbath surfaces at 22°C.

The association between genetic ability and phenotypic expression of biofilm has been described. Previous work has shown that the *fimH* gene, which encodes for the

expression of type 1 fimbriae, was carried by all *E. coli* O157:H7 strains evaluated, but none produced type 1 fimbriae (Biscola, Abe, and Guth, 2011). This is likely due to the fact that *E. coli* O157:H7 strains lack the ability to express type 1 fimbriae because of a deletion within the regulatory region that controls type 1 fimbriae expression (Li, Koch, and Cebula, 1997). Therefore, even though *fimH* is present in the genome of *E. coli* O157:H7, it likely does not contribute to biofilm formation.

Curli, the long aggregative fimbriae common in many *Enterobacteriaceae*, are known to contribute to biofilm formation by promoting adhesion of bacteria to both biotic and abiotic surfaces (Zogaj *et al*., 2003). Curli is primarily composed of CsgA subunit proteins, encoded by the *csgA* gene, that self-polymerize into a β-sheet-rich amyloid fiber (Chapman *et al*., 2002). Although the prevalence of *csgA* appears to be high, and in one study, was carried by all of the *E. coli* O157:H7 strains, expression of curli fimbriae appears low and only occurred in 28% of the *E. coli* O157:H7 strains in the study (Biscola, Abe, and Guth, 2011). Although CsgD regulates curli biosynthesis by controlling transcription of the *csgBAC* operon (Romling, 2005), both *csgA* and *csgD* are needed for the expression of curli fimbriae (Barnhart and Chapman, 2006).

Temperatures below 30°C have been shown to promote curli gene expression (Arnqvist *et al*., 1992; Olsen, Jonsson, and Normark, 1989; Barnhart and Chapman, 2006), which may explain why more *E. coli* O157:H7 isolates in our study produced more dense biofilms at 22°C than at 37˚C or 42˚C. Other environmental conditions may also stimulate curli expression, such as nutrient limitation and oxygen tension (Gerstel and Romling, 2001; Romling *et al*., 1998). In a footbath, the availability of nutrients and oxygen may vary depending on how often the footbath is used. If the footbath receives frequent traffic, the

amount of organic contamination and oxygen in the footbath would increase, possibly decreasing the likelihood of curli expression by *E. coli* O157:H7 in the footbath. Curli expression also appears to be strain-dependent among *E. coli* O157:H7 isolates due to the complex regulatory network directing curli synthesis (Carter *et al*., 2011; Carter *et al*., 2016). Isolates with mutations in the promoter of *csgD* have been linked to increased curli biogenesis due to upregulation in the transcription of the *csgA* and *csgB* genes (Uhlich, Keen, and Elder, 2001). All seven of the *E. coli* O157:H7 isolates used in this study were shown to have the *csgD* and *csgA* genes required for curli synthesis, even though not all of them produced prominent biofilm. Other genetic and regulatory factors on a strain-specific level may be influencing curli expression in these *E. coli* O157:H7 isolates to promote biofilm formation. Further work is needed determine the levels of curli production in these isolates during biofilm formation on polystyrene plastic and rubber footbaths, and the effect curli production has on biofilm formation.

The *pgaABCD* operon encodes gene products necessary for the synthesis of PGA adhesins. PgaC is the primary glycosyltransferase that synthesizes PGA, while PgaA serves as an export protein to transfer PGA to the ECM (Itoh *et al*., 2008). All of the gene products from the *pgaABCD* operon are necessary for optimal biofilm formation in *E. coli* K-12 under a variety of growth conditions (Itoh *et al*., 2008; Wang *et al*., 2004). All seven of the *E. coli* O157:H7 strains used in this study had both the *pgaA* and *pgaC* genes needed for biofilm formation. However, since not all strains produced biofilm, it is possible that other genetic and regulatory factors may influence the expression of these genes, or that other gene products may be needed to form optimal biofilms.

Additionally, the seven *E. coli* O157:H7 strains used in this study were negative for *bfp* and *sfa* genes, which encode products that may aid in bacterial adherence to surfaces (Naves *et al*., 2008; LeBlanc, 2003). Still, these genes are not required for biofilm synthesis. The *sfa* gene encodes S fimbrial adhesins, which assist bacteria in adhering to solid surfaces (Naves *et al*., 2008). A previous study found that 93% of *E. coli* strains belonging to O1, O2, O4, O6, O7, O14, O15, O18, O22, O75, and O83 possessed *sfa* operons, versus only 32% of strains belonging to other serogroups (Blanco *et al*., 1997). The *bfp* gene codes for a membranous cellular extension called a bundle-forming pillus (BFP) that is used to attach to host cells and form microcolonies. Bundle-forming pilli are used by enteropathogenic *E. coli* (EPEC) to colonize the ileum (LeBlanc, 2003). Enterohemorrhagic *E. coli* (EHEC), such as *E. coli* O157:H7, have not been shown to carry the *bfp* gene, and are therefore unlikely to colonize the ileum. They use other virulence factors to adhere to cells in the colon (Nataro and Kaper, 1998; Vallance and Finlay, 2000). As expected, none of the *E. coli* O157:H7 strains in this study had *bfp* or *sfa* genes (Table 2.1).

Previous work has shown that *E. coli* strains growing in biofilms are less susceptible to antimicrobials that non-biofilm forming strains (Ranjith *et al*., 2017). It has also been hypothesized that the likelihood of horizontal gene transfer may be increased in biofilms, thus increasing the potential for transfer of antimicrobial resistance genes between strains growing within a biofilm (Wang *et al*., 2016). All of the *E. coli* O157:H7 strains in this study displayed MICs of 4 to 8 µg/mL towards amoxicillin/clavulanate and chloramphenicol, less than 0.015 µg/mL of ciprofloxacin, 0.5 to 1 µg/mL of gentamicin, and less than 0.12 µg/mL of trimethoprim/sulfamethoxazole. Six out of seven strains displayed MICs of 2-4 µg/mL to ampicillin, while one strain (171F) had an MIC of greater than 32 µg/mL towards ampicillin.

This susceptibility profile is unremarkable considering high levels of *E. coli* resistance to ampicillin have been previously observed (El-Shatoury *et al*., 2015). Therefore, no link between antimicrobial resistance and biofilm formation was revealed in this study.

Because the biomass and $\log_{10}$(CFU/mL) values were categorized with some considered "prominent" in this study, there were instances where strains were not statistically different from other strains considered non-prominent; therefore, our categorical description of "prominent" biofilms is not perfect, but is still useful in denoting how many strains appeared to form more dense biofilms under certain conditions. An additional consideration for this study is that footbaths had to be cut into pieces to perform biofilm assays. This leaves the possibility of inconsistencies between piece sizes and formation of small abrasions in the rubber that could affect bacterial binding and biofilm formation. However, replication and random use of footbath pieces minimized this potential effect. Although this assay did not perfectly mimic an actual footbath setting, it allowed for well described potential of biofilm on rubber material. Because the *E. coli* O157:H7 strains were induced to form biofilm in M9 minimal media, it does not reflect what would be seen in all footbaths. Other components, such as the presence of disinfectants and organic matter in footbaths, may affect biofilm formation on their surfaces.

In conclusion, our data suggests that *E. coli* O157:H7 may begin to form biofilm on rubber footbath surfaces at 22°C within 24 hours of growth. It is not known how early these biofilms appeared. Although the biofilms were not dense, if these biofilms could confer resistance or tolerance to disinfectants within the footbath, they would be a risk for pathogen persistence and transmission in an animal interaction setting. Specific footbath recommendations should take into account the biofilm potential of fecal pathogens given the

temperature and frequency with which they are used. It may be necessary to replace the disinfectant solution and thoroughly clean footbaths by scrubbing the surface on a more regular occasion to prevent the formation of biofilm and retain efficacy of the footbath. Considering no biofilm formation was apparent at 37°C or 42°C on rubber footbath surfaces, it may be desirable to clean footbaths on a more regular occasion when temperatures are lower to prevent the formation of biofilm. Further work is needed to determine if *E. coli* O157:H7 and other bacteria form biofilms on footbaths in a practical setting, and if the formation of biofilms on footbath surfaces decreases susceptibility of the bacteria to footbath disinfectants and increases bacterial viability within the footbath.

2.4 Materials and Methods

2.4.1 Bacterial strains

Escherichia coli O157:H7 isolates were previously obtained from goat feces, hides, and carcasses at a USDA-inspected processing plant in North Carolina (Jacob *et al*., 2013). Seven genetically distinct *E. coli* O157:H7 isolates, as determined by pulsed-field gel electrophoresis, and the biofilm-positive control strain *E. coli* ATCC 25922 (Naves *et al*., 2008; Cremet *et al*., 2013) were selected for biofilm analysis. Three of the *E. coli* O157:H7 strains were isolated from feces, two were from carcasses, and two were from hides. All strains were stored in tryptic soy broth with 25% glycerol at -80˚C and sub-cultured onto blood agar from freezer stocks for each assay.

2.4.2 Biofilm potential on polystyrene plastic

The seven *E. coli* O157:H7 strains were inoculated individually in 2 mL of lysogeny broth (LB; Sigma-Aldrich, St. Louis, MO) and incubated overnight at 37˚C. The concentrations of the overnight cultures were adjusted to approximately 2 McFarland Standards (McF, approximately 6.0×10^8 CFU/mL) using a DensiCHEK plus (BioMerieux). Ten microliters of each concentration-adjusted culture was added to 90 µL of M9 minimal media (Sigma-Aldrich) in 96-well plates (Thermo Fisher Scientific, Rochester, NY). Polystyrene MBEC biofilm inoculator peg lids (Innovotech, Edmonton, Canada; Figure C) were placed on the 96-well plates, and the plates were incubated with shaking at 22˚C, 37˚C, and 42˚C for 24, 48, and 72 hours. The media was replaced in the 96-well plates every 24 hours to account for evaporation. Each sample was evaluated in triplicate, and the assay was performed two different times.

To measure the relative amount of biomass in the biofilms attached to the polystyrene pegs, the pegs were dipped three times in 150 µL of sterile phosphate-buffered saline (PBS; Fisher BioReagents, Fair Lawn, NJ) to remove loosely-bound planktonic cells, and then stained for 15 minutes in 150 µL of a 1:5 solution of crystal violet (VWR, Randnor, PA) and PBS. The pegs were then rinsed five more times by dipping in 150 µL of sterile PBS to remove excess crystal violet. The crystal violet bound to the biofilms was eluted by soaking the pegs in 150 µL of ethanol for 15 minutes. A Synergy 2 microplate reader (BioTek Instruments) was used to measure the absorbance of the ethanol with eluted crystal violet in each well at 550 nm.

To estimate the number of viable cells in the biofilms, the pegs were rinsed by dipping three times in 150 µL of sterile PBS to remove loosely bound planktonic cells, and

then the peg lid was placed on a new 96-well plate containing 150 µL of sterile PBS in each well. The plate was sonicated in a 1.9 L ultrasonic bath (Fisher Scientific) on low for 5 minutes to disrupt the biofilms. Each replicate sample was joined and serially diluted ten-fold in sterile PBS. The dilutions were spotted in 20 µL aliquots three times on tryptic soy agar (TSA; Becton Dickinson, Sparks, MD), and the TSA plates were incubated overnight at 37˚C. Colonies were counted to calculate the CFUs/mL of viable *E. coli* O157:H7 in the biofilms.

2.4.3 Biofilm potential on rubber footbaths

A rubber footbath (Wearwell, Smyrna, TN) was cut into 3 cm × 3 cm pieces (Figure C), and the pieces were autoclaved at 121˚C for 15 minutes to sterilize. Concentration-adjusted cultures of the *E. coli* O157:H7 strains were prepared as described above, and 0.5 mL of each culture was added to 4.5 mL of M9 in 6-well plates (VWR), along with one footbath piece per well. The plates were incubated at 22˚C, 37˚C, and 42˚C for 24 hours. Each sample was evaluated in duplicate, and the assay was performed two different times.

To determine the amount of biomass in the biofilms attached to the surface of the rubber footbath pieces, the pieces were removed from the media using sterile forceps and dipped three times in 6 mL of sterile PBS to remove loosely bound cells. The pieces were stained for 15 minutes in 6 mL of a 1:5 solution of crystal violet with PBS, then rinsed five more times in 6 mL of PBS. The crystal violet was eluted from the biofilms by soaking the pieces in 6 mL of ethanol for 15 minutes. The absorbance of the ethanol with eluted crystal violet was performed as previously described.

The number of viable cells in the biofilms attached to the rubber footbath pieces was estimated by rinsing the pieces in 6 mL of sterile PBS three times to remove loose cells. The pieces were placed in new 6-well plates with 6 mL of sterile PBS per well, and the biofilms were disrupted by sonicating the plates on low for 5 minutes. The samples were joined, diluted, and spot plated on TSA as previously described. Colonies were counted to calculate the number of CFUs/mL recovered from the biofilms after sonication.

2.4.4 PCR characterization

Conventional PCR was used to characterize the seven *E. coli* O157:H7 strains for biofilm-associated genes. Gene targets included *bfp* (Gunzburg, Tornieporth, and Riley, 1995) (bundle forming pilus), *fimH* (Moulin-Schouler *et al*., 2006) and *sfa* (Chen *et al*., 2003) (fimbrial adhesins), *csgA* (Cremet *et al*., 2013) and *csgD* (Cremet *et al*., 2013) (curli fimbriae), *pgaC* (Cremet *et al*., 2013) (adhesin polysaccharide), and *pgaA* (Cremet *et al*., 2013) (adhesin polysaccharide export). All PCRs were performed by suspending a colony of bacteria in 100 µL of sterile water. The suspensions were boiled for 25 minutes to obtain DNA. The reaction conditions are listed in Table 2.3. The DNA was added to PCR tubes, along with the appropriate primers (Table 2.3) and either Phusion High Fidelity DNA Polymerase (Thermo Scientific, Fisher) or GoTaq Master Mix (Promega, Fisher). All reactions were initially denatured at 98˚C for 30 seconds, followed by 30 cycles of denaturing at 98˚C for 30 seconds, annealing at 52˚C for 30 seconds, and extension at 72˚C for 30 seconds. The reactions were completed with a final extension at 72˚C for 5 minutes. Products were separated using the QIAxcel Advanced capillary electrophoresis device (QIAGEN) and visualized using the QIAxcel ScreenGel software.

Table 2.3. PCR reagents for amplification of biofilm-related genes in *Escherichia coli* O157:H7.

Reaction	Reagent	Amount per reaction (µL)	Primer Sequence
bfp	dH_2O	9.5	
	bfp-Fwd[a]	1	AAT GGT GCT TGC GCT TGC TGC
	bfp-Rev[a]	1	GCC GCT TTA TCC AAC CTG GTA
	GoTaq	12.5	
pgaA and *fimH*	dH_2O	9.5	
	pgaA-Fwd[b]	0.5	AGG GGA CTG CGC ATT GAT TAC
	pgaA-Rev[b]	0.5	GTT CAC GTT CGA CAA CAT CG
	fimH-Fwd[c]	0.5	GAT CTT TCG ACG CAA ATC
	fimH-Rev[c]	0.5	CGA GCA GAA ACA TCG CAG
	Phusion	12.5	
csgD, *csgA*, and *sfa*	dH_2O	8.5	
	csgD-Fwd[b]	0.5	ACA GCT CTC TTG CAG CAC CT
	csgD-Rev[b]	0.5	ACG GGT AAT CTT CAG GCG TA
	csgA-Fwd[b]	0.5	GGG CTC AGA TGA TGA CAG CTC AAT C
	csgA-Rev[b]	0.5	CGG CTT CCA CTG ATC AAG AGT AG
	sfa-Fwd[d]	0.5	CTC CGG AGA ACT GGG TGC ATC TTA C
	sfa-Rev[d]	0.5	CGG AGG AGT AAT TAC AAA CCT GGC A
	Phusion	12.5	
pgaC	dH_2O	10.5	
	pgaC-Fwd[b]	0.5	ATG ATT AAT CGC ATC GTA TCG
	pgaC-Rev[b]	0.5	CAT CGG TTC CAC AAT ATA TG
	GoTaq	12.5	

[a] (Gunzburg, Tornieporth, and Riley, 1995)
[b] (Cremet *et al*., 2013)
[c] (Moulin-Schouleur *et al*., 2006)
[d] (Chen *et al*., 2003)

2.4.5 Antimicrobial susceptibility

The antimicrobial susceptibility of the seven *E. coli* O157:H7 strains were determined by microbroth dilution using the automated Sensititre system (TREK Diagnostic Systems, Thermo Scientific). One colony of each strain was suspended in sterile saline to achieve a 0.5 McF standard, and 10 µL was inoculated into Mueller-Hinton broth (MHB; Thermo Scientific). An autodispenser added 100 µL to each well of a Sensititre MIC plate (Thermo Scientific). Plates were evaluated automatically after 18 hours. The lowest concentration of antimicrobial in which no turbidity was measured was determined to be the minimum inhibitory concentration (MIC), and interpretations were derived from the Sensititre software.

2.4.6 Statistical analysis

SAS 9.4 software was used to model the biomass and $\log_{10}$(CFU/mL) data using a mixed linear model where interactions among temperature, time, and strain were used as factors. Least square means were estimated and compared between strains by time and temperature, and adjusted p-values were obtained using Tukey's method.

To determine which strains formed prominent biofilms under which specific conditions, outliers were excluded from the biomass and $\log_{10}$(CFU/mL) data (quartile 3 plus 1.5 × inter quartile range and quartile 1 minus 1.5 × inter quartile range), and the ranges of the data sets were separated into six sections. Each section was assigned a rank (0 for the lowest values, 5 for highest values), and ranks were given to each value. Upper outliers were assigned rank 5. There were no lower outliers. Biomass and $\log_{10}$(CFU/mL) values that were ranked 4 or 5 were considered prominent.

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CHAPTER 3

Susceptibility of *Escherichia coli* O157:H7 to disinfectants *in vitro* and in simulated footbaths

Abstract

Escherichia coli O157:H7 is a human pathogen that can cause severe gastrointestinal disease. Naturally existing in the intestines of ruminants, *E. coli* O157:H7 may be spread through direct contact with animals and their feces at agritourism or livestock interaction events. Footbath use may be one pathogen reduction strategy to decrease transmission of *E. coli* O157:H7. The efficacy of chemical disinfectants in footbaths is not well understood, as the disinfectant type and organic contamination may affect footbath efficacy. Our objective was to determine the susceptibility of *E. coli* O157:H7 towards commonly used disinfectants *in vitro* and within simulated footbaths. The minimum inhibitory concentration (MIC) and time-to-kill was determined *in vitro* for genetically distinct *E. coli* O157:H7 strains for five disinfectant compounds (didecyl dimethyl ammonium chloride (DDAC), glutaraldehyde, hydrogen peroxide, phenol, and sodium hypochlorite). Time-kill assays were also performed within simulated footbaths at 22˚C, 37˚C, and 42˚C with and without organic contamination using three commercial disinfectants (0.26% Clorox® bleach, 0.034% Virex® II 256, and 1% Virkon™ S). The MICs of each disinfectant towards *E. coli* O157:H7 were 3.2, 625, 20, 2500, and 320 ppm of DDAC, glutaraldehyde, hydrogen peroxide, phenol, and sodium hypochlorite, respectively. At 2 × MIC, *E. coli* O157:H7 was killed on contact with glutaraldehyde, by 20 minutes with DDAC and sodium hypochlorite, and by 4 hours with phenol and hydrogen peroxide. In the simulated footbaths, all three commercial disinfectants killed the bacteria on contact in the absence of organic contamination, but viable *E. coli*

O157:H7 was recovered from organically contaminated Clorox® bleach and Virex® II 256 footbaths. No *E. coli* O157:H7 was recovered from the Virkon™ S footbaths after 10 minutes. This study highlights the importance of choosing an appropriate disinfectant and frequently cleaning contaminated footbaths to prevent the spread of potential pathogens and to retain efficacy of the footbath.

3.1 Introduction

Escherichia coli O157:H7 is a human pathogen that naturally exists within the digestive tracts of ruminants. Previous studies have found that *E. coli* O157:H7 prevalence in cattle ranges from 9.2 to 20.3% (Alam and Zurek, 2006; Reinstein *et al*., 2009; Walker *et al.*, 2010), and from less than 5% to as high as 11% in goat feces (Keen *et al*., 2006; Mersha *et al*., 2010; Jacob *et al*., 2013). Many studies have shown associations between diet, season, production system, and *E. coli* O157:H7 prevalence in ruminants (Hovde *et al.*, 1999; Edrington *et al.*, 2006; Gansheroff and O'Brien, 2000). *Escherichia coli* O157:H7 was first identified as a pathogen in 1982, and it has been found to cause several diseases including bloody diarrhea, thrombocytopenic purpura, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Doyle, 1991; Mead and Griffin, 1998; Wang *et al*. 2012). Hemolytic uremic syndrome is one of the most severe complications of *E. coli* O157:H7 infections, potentially leading to kidney failure in young children, the elderly, and the immunocompromised (Durso, Bono, and Keen, 2005).

Since *E. coli* O157:H7 can be shed asymptomatically in the feces of many ruminants, it is possible to acquire *E. coli* O157:H7 through interacting with these animals, particularly among people who are not accustomed to proper handling procedures. Of major concern is the risk of infection to children that are visiting petting zoos, farms, and agritourism events with these animals. Outbreaks of *E. coli* O157:H7 have been associated with agritourism events in many states across the United States, including North Carolina, Florida, Arizona, Minnesota, and recently in Connecticut (CDC, 2006; Saulo, 2013, Laughlin *et al.*, 2016).

Biosafety and pathogen control strategies such as handwashing should be taken to prevent *E. coli* O157:H7 infections at agritourism events. Previous work has shown that hand

hygiene was only practiced by 37% of observed visitors at petting zoos (Erdozain *et al.*, 2012), suggesting that mitigation strategy alone is insufficient to protect against infection at public-livestock interactions. Footbaths may be used as a viable biosafety measure to reduce the risk of pathogen transmission at these events. Microorganisms have previously been shown to survive for days or weeks in dirt that is stuck to the bottom of shoes (McCrea and Bradley, 2008). Footbaths, which are disinfectant-filled mats or pans placed at the entrances and exits of animal habitats, are frequently used on farms to clean shoes and boots when leaving a soiled animal habitat. Several studies have shown that footbath use may decrease viable bacterial load on shoes and floors, though efficacy can be variable depending on the type of disinfectant in the footbath, the presence and amount of contamination in the footbath, temperature, and exposure to sunlight (Hornig *et al.*, 2016; Morley *et al.*, 2005; Allen *et al.*, 2010; Dunowska *et al*., 2006; Hartmann, Dusick, and Young, 2013; McCrea and Bradley, 2008; USDA, 2002). While multiple studies have determined the persistence of bacteria on the soles of boots and surfaces of floors after stepping through footbaths, few studies have examined bacterial persistence within footbaths.

General guidelines recommend that footbaths should be cleaned and refilled with freshly prepared disinfectant solution daily, the footbath should be used both when entering and exiting an animal habitat, and that both employees and visitors to the farm should use the footbaths (Gernat, 2004). Recommended disinfectants for footbaths include phenols, iodophors, hypochlorites, quaternary ammonium compounds (QACs), oxidizing agents, compound cresol, and formals (Gernat, 2004; Jeffrey, 1997). According to a survey of 31 veterinary teaching hospitals, the most common disinfectants used in footbaths were

quaternary ammonium products (42%), phenolics (39%), hypochlorite solutions (39%), and peroxygen disinfectants (19%) (Morley *et al*., 2005).

Our objective was to determine the efficacy and length of time needed for laboratory-grade and commercially available disinfectants to kill *E. coli* O157:H7 *in vitro* and within a simulated footbath, respectively. Previous work has shown that the efficacy of disinfectants can be reduced in the presence of organic matter, such as on the surface of produce or in a lettuce or tomato extract homogenate (Rodgers *et al.*, 2003; Shen *et al*., 2012). Therefore, we also aimed to determine the viability and persistence of *E. coli* O157:H7 within a simulated footbath environment at different temperatures with and without the presence of organic contamination. If *E. coli* O157:H7 is capable of persisting within contaminated footbaths for extended periods of time, footbaths may not be a practical biosafety measure to decrease the risk of *E. coli* O157:H7 spread and infection, or footbath guidelines may need to be changed to improve their efficacy.

3.2 Materials and Methods

3.2.1 Bacterial strains

E. coli O157:H7 strains previously obtained from goat feces, hides, and carcasses at a USDA-inspected processing plant in North Carolina (Jacob *et al*., 2013) were used in these experiments. Seven genetically distinct strains were selected to identify the minimum inhibitory concentrations (MICs) towards disinfectant compounds. Three of these strains (171F, 202H, and 208H) were selected for *in vitro* time-kill analyses, and three different strains (217C, 229F, and 230C) were used in the time-kill analyses in simulated footbaths. The three *E. coli* O157:H7 strains used in simulated footbaths were made resistant to 50

µg/mL of nalidixic acid by inoculating the strains in 2 mL of Mueller-Hinton broth (MHB; Becton Dickinson, Sparks, MD) supplemented with 1 µg/mL of nalidixic acid. The cultures were incubated overnight at 37°C and streaked onto sorbitol MacConkey agar (Criterion, Hardy Diagnostics, Santa Maria, CA) supplemented with 0.5 µg/mL of cefixime and 2.5 µg/mL of tellurite (CT-SMAC). The plates were incubated overnight at 37°C, and three to five colonies were inoculated in 2 mL of MHB with double the concentration of nalidixic acid as the previous culture. This process was repeated until 50 µg/mL of nalidixic acid was reached. Isolates were confirmed to be *E. coli* O157:H7 by PCR after inducing resistance.

3.2.2 Determining the minimum inhibitory concentration

Microbroth determination of the minimum inhibitory concentration was conducted according to Clinical Laboratory Standards Institute Guidelines (CLSI, 2015). The MIC of five laboratory-grade disinfectants (1% didecyl dimethyl ammonium chloride (DDAC; Sigma-Aldrich, St. Louis, MO), 25% glutaraldehyde (Fisher Chemical, Fair Lawn, NJ), 30% hydrogen peroxide (Fisher Chemical), 8% phenol (Sigma-Aldrich), and 4% sodium hypochlorite (Sigma-Aldrich)) towards seven strains of *E. coli* O157:H7 (171F, 202H, 208H, 217C, 226F, 229F, and 230C) were determined. Phenol and DDAC were dissolved in sterile deionized water to produce 8% and 1% solutions, respectively. Strains were streaked on blood agar (Remel, Lenexa, KS) and incubated overnight at 37°C. One colony was selected for each isolate and inoculated in 2 mL of MHB or M9 minimal media. M9 minimal media was prepared by combining 100 mL of 10× M9 salts (Sigma-Aldrich) with 1 mL of 1 M magnesium sulfate (Sigma-Aldrich), 10 mL of 20% glucose (Sigma-Aldrich), 0.2 mL of 0.5 M calcium chloride (Sigma-Aldrich), 0.5 mL of 1 M Thiamine (Sigma-Aldrich), and 888.3

mL of deionized water per 1 liter of media. The media was filtered to sterilize. The MHB and M9 cultures were incubated at 37˚C, and the bacterial concentrations were periodically determined using a DensiCHEK plus (BioMerieux). Incubation was concluded once the cultures reach an absorbance of 0.5 McFarland Standard (approximately 1.5×10^8 CFU/mL; approximately 3 hours for MHB and 6 hours for M9). Twenty microliters of each enrichment was diluted 1:100 in 2 mL of MHB or M9 to produce a concentration of approximately 1.5×10^6 CFU/mL. The disinfectants were diluted in 1 mL of MHB or M9 such that their concentration was double that of the highest concentration assayed (3.2 ppm DDAC, 5000 ppm glutaraldehyde, 80 ppm hydrogen peroxide, 20,000 ppm phenol, and 320 ppm sodium hypochlorite). Fifty microliters of MHB or M9 was added to each well in columns 2 through 12 of a 96-well plate, and 50 µL of the MHB or M9 with disinfectant was added to columns 1 and 2. 50 µL was serially diluted from columns 2 through 11, and 50 µL was discarded from column 11 so that all wells contained the same volume of media. Column 12 was left with no disinfectant as a positive control. Fifty microliters of the bacterial cultures were added to each well, giving a final bacterial concentration of approximately 7.5×10^5 CFU/mL per well. The 96-well plates with MHB were incubated for 24 hours at 37˚C. The plates with M9 were incubated for 48 hours at 37˚C due to slower growth of *E. coli* O157:H7 in minimal media than in MHB. After incubation, growth was recorded in the wells, and the lowest concentration not to show turbidity was determined to be the MIC of the disinfectant.

3.2.3 In vitro *time-kill assays*

One colony of three individual *E. coli* O157:H7 strains (171F, 202H, and 208H) were inoculated in 2 mL of MHB and incubated at 37˚C. The bacterial concentration was

determined using a DensiCHEK plus until the cultures reached an absorbance of 0.5 McFarland. Forty microliters of each enrichment was diluted 1:100 in 4 mL of MHB to give a concentration of approximately 1.5×10^6 CFU/mL. Disinfectants were diluted in 1 mL of MHB to achieve concentrations of 1 ×, 2 ×, and 4 × the MIC as determined for each disinfectant. One milliliter of the diluted bacterial culture was added to 1 mL of the MHB with disinfectant so that the final disinfectant concentrations were 0.5 ×, 1 ×, and 2 × MIC for each disinfectant (Table 3.1) and the final bacterial concentration was approximately 7.5×10^5 CFU/mL in each well. A 100 µL aliquot was removed from each culture and serially diluted ten-fold in sterile phosphate buffered saline (PBS; Fisher BioReagents, Fair Lawn, NJ). Twenty microliters of each dilution was spotted onto tryptic soy agar (TSA; Becton Dickinson) in triplicate to confirm the starting concentration. The time-kill cultures were incubated at 37˚C. One-hundred microliter aliquots were removed at 10 min, 20 min, 30 min, 1 hr, 4 hr, and 24 hr. Aliquots were serially diluted ten-fold in sterile PBS and 20 µL of each dilution was spotted in triplicate onto TSA and incubated overnight at 37˚C. Colonies were counted to determine the bacterial concentration at each time interval.

Table 3.1. Disinfectant concentrations used for time-kill assays at the corresponding minimum inhibitory concentration (MIC).

Disinfectant Compound	0.5 × MIC	1.0 × MIC	2.0 × MIC
Didecyl dimethyl ammonium chloride	1.6 ppm	3.2 ppm	6.4 ppm
Glutaraldehyde	313 ppm	625 ppm	1250 ppm
Hydrogen peroxide	20 ppm	40 ppm	80 ppm
Phenol	1250 ppm	2500 ppm	5000 ppm
Sodium hypochlorite	160 ppm	320 ppm	640 ppm

3.2.4 Goat feces collection and E. coli *O157:H7 screening for footbath assays*

Approximately 20 grams of fresh goat feces was collected before each footbath assay. The feces was homogenized using a mortar and pestle. One gram of feces was added to 9 mL of gram negative broth (Becton Dickinson) supplemented with 0.5 mg/L cefixime, 10 mg/L cefsulodin, and 8 mg/L vancomycin (GNccv) and incubated for six hours at 37˚C. Immunomagnetic separation was performed using 20 µL of anti-*E. coli* O157 dynabeads (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA) according to manufacturer instructions. Beads were washed three times in 100 µL of sterile PBS, and a 50 µL aliquot was spread on CT-SMAC and incubated overnight at 37˚C. The plate was observed for gray colonies, and the feces were confirmed negative for *E. coli* O157:H7 prior to their use.

3.2.5 Footbath time-kill assays

A rubber footbath (Wearwell, Smyrna, TN) was cut into 9 cm × 12 cm pieces and laid into empty 9 cm × 12 cm plastic boxes to simulate a footbath. One gram of pine wood shavings (Tractor Supply Company, Brentwood, TN) was added to half of the footbaths and all footbaths were autoclaved to sterilize. Two grams of goat feces were added to each footbath with pine wood shavings.

One colony from each of three strains of *E. coli* O157:H7 strains made resistant to 50 µg/mL of nalidixic acid were inoculated in 17 mL of MHB and incubated at 37˚C until the bacterial density reached 0.5 McFarland. Footbaths were prepared either by adding 100 mL of sterile deionized water to empty footbaths or footbaths with organic matter, or 100 mL of sterile deionized water with disinfectant to empty footbaths or footbaths with organic matter. The footbaths with disinfectant had one of three commercially available disinfectants at the

manufacturer's recommended concentration (0.26% Clorox® bleach (Clorox® Professional Products Company, Oakland, CA), 0.034% Virex® II 256 (Diversy, Charlotte, NC), and 1% Virkon™ S (Virkon™, The Chemours Company, Wilmington, DE)). In order to determine the efficacy of disinfectants in footbaths at various temperatures, the footbaths were incubated at 22˚C , 37˚C, and 42˚C for one hour to equilibrate the temperature of the water in the footbaths, after which 1 mL of bacterial culture was added to each footbath. After manually mixing the footbath, 100 µL aliquots were taken at 0 minutes, 15 minutes, 30 minutes, 1 hour, 4 hours, and 24 hours. The aliquots were added to 900 µL of Dey-Engley Neutralizing broth (D/E broth; Criterion, Hardy Diagnostics) and incubated at room temperature for 15 minutes. The samples were serially diluted ten-fold and plated onto CT-SMAC supplemented with 50 µg/mL of nalidixic acid (CTN-SMAC). The plates and D/E samples were incubated overnight at 37˚C, and gray colonies were counted to determine the concentration of *E. coli* O157:H7 at each time point. If no colonies were present on the plates, a 10 µL aliquot of the incubated D/E sample was plated onto CTN-SMAC to determine if the bacterial concentration was below the limit of direct detection.

3.2.6 Study analysis

The concentration (CFU/mL) of each strain were averaged for each time point to produce a mean CFU/mL value, which were reported as $1og_{10}$(CFU/mL). The bacteria was determined to be "killed" if the bacterial concentration decreased by 3 log_{10}(CFU/mL), or a 99.9% reduction (CLSI, 2015). In some cases where bacterial concentrations decreased by 3 log_{10}(CFU/mL), but bacteria were still recovered, the bacteria were considered substantially reduced, but still detectable. For the footbath time-kill assays, in cases where bacterial

concentrations were reduced below the limit of detection, but viable bacteria were still recovered from the enriched aliquots, the bacterial concentration was considered to be the lowest concentration at which CFUs could be detected (1.67×10^2 CFU/mL).

3.3 Results

3.3.1 Minimum inhibitory concentrations

Strains behaved similarly in their MICs toward the five laboratory-grade disinfectant compounds (DDAC, glutaraldehyde, hydrogen peroxide, phenol, and sodium hypochlorite). The median MIC value of all strains for each disinfectant in MHB and M9 are listed in Table 3.2. In MHB, DDAC and hydrogen peroxide had the lowest MICs (3.2 ppm and 40 ppm, respectively), while phenol had the highest MIC (2500 ppm). When the assay was repeated for each disinfectant in M9 media, all MICs were reduced, though the MICs of some disinfectants were reduced more than others. The MIC of DDAC and phenol were reduced two-fold (3.2 to 1.6 ppm and 2500 to 1250 ppm, respectively) in M9, the MIC of hydrogen peroxide was reduced four-fold (40 to 10 ppm), glutaraldehyde's MIC was reduced by a factor of 125 (625 to 5 ppm), and the MIC of sodium hypochlorite was reduced by a factor of 256 (320 to 1.25 ppm).

Table 3.2. Median minimum inhibitory concentration (MIC) values of laboratory-grade disinfectant compounds for *Escherichia coli* O157:H7 grown in Mueller-Hinton broth (MHB) and M9 media.

Disinfectant	Median MIC in MHB	Median MIC in M9	Fold Decrease
Didecyl dimethyl ammonium chloride	3.2 ppm	1.6 ppm	2
Glutaraldehyde	625 ppm	5 ppm	125
Hydrogen peroxide	40 ppm	10 ppm	4
Phenol	2500 ppm	1250 ppm	2
Sodium hypochlorite	320 ppm	1.25 ppm	256

3.3.2 In vitro *time-kill assays*

For all disinfectants and time points, 0.5 × MIC did not achieve bactericidal activity, but at 1 × MIC and 2 × MIC, either the concentrations of *E. coli* O157:H7 were static or the bacteria were killed within 24 hours of exposure. Figure 3.1 shows the average $\log_{10}$(CFU/mL) of *E. coli* O157:H7 at each time point after exposure to the five disinfectant compounds at 0.5 ×, 1 ×, and 2 × MIC.

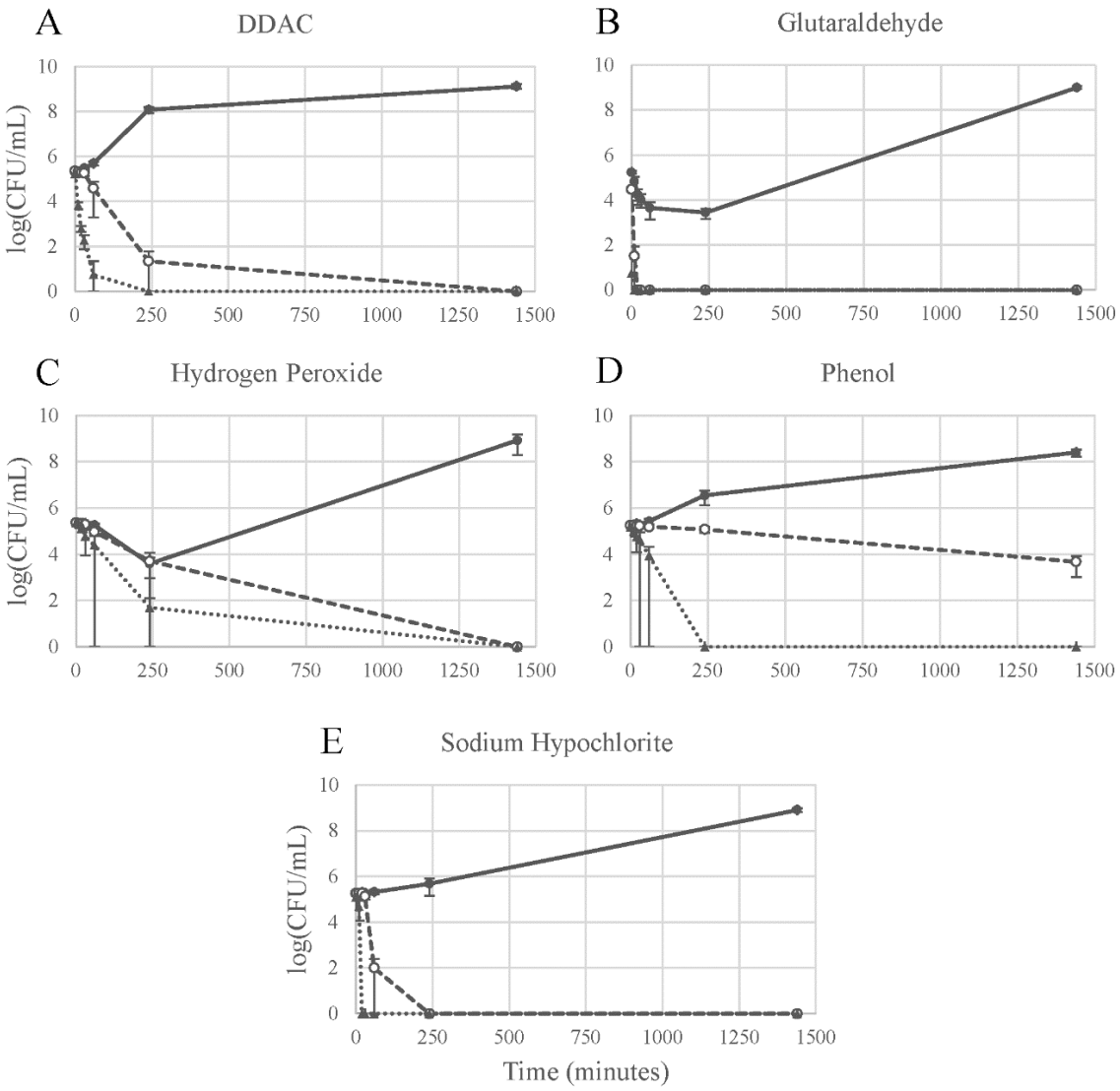

Figure 3.1. Viable *Escherichia coli* O157:H7 concentrations over time when exposed to five disinfectant compounds at concentrations representing 0.5, 1, and 2 × the minimum inhibitory concentration (MIC). Three strains of *E. coli* O157:H7 were individually exposed to 0.5 (filled circle, solid line), 1.0 (open circle, dashed line), and 2 × MIC (filled triangle, dotted line) of didecyl dimethyl ammonium chloride (A), glutaraldehyde (B), hydrogen peroxide (C), phenol (D), and sodium hypochlorite (E). Concentrations were enumerated at 0 min, 10 min, 20 min, 30 min, 1 hr, 4 hr, and 24 hr, and the concentrations for the three strains were averaged.

At 0.5 × MIC of DDAC, *E. coli* O157:H7 gradually increased in number to a final concentration of 9.12 $\log_{10}$(CFU/mL) This was not observed when DDAC exposure was at 1 × and 2 × MIC, where the bacteria were killed by 4 hours and 20 minutes, respectively.

When exposed to 0.5 × MIC of glutaraldehyde, the *E. coli* O157:H7 initially decreased in concentration for the first hour of exposure, but then began to increase after 4 hours to a final concentration of 9.01 $\log_{10}$(CFU/mL). At 1 × MIC of glutaraldehyde, the bacteria were killed by 10 minutes, and at 2 × MIC, the bacteria were killed on contact. At 0.5 × MIC of hydrogen peroxide, *E. coli* O157:H7 decreased in concentration for the first 4 hours, but then increased between the 4 hours and 24 hours to a final concentration of 8.93 $\log_{10}$(CFU/mL). A three log reduction and bactericidal activity was achieved by 24 hours at 1 × MIC and by 4 hours at 2 × MIC. When exposed to phenol at 0.5 × and 1.0 × MIC, bactericidal activity was not reached at any time point. *Escherichia coli* O157:H7 increased in concentration throughout exposure to 0.5 × MIC (final concentration of 8.40 $\log_{10}$(CFU/mL)), and the bacterial concentration remained relatively stable throughout the course of the experiment at 1.0 × MIC (final concentration of 3.68 $\log_{10}$(CFU/mL). At 2 × MIC of phenol, bactericidal activity was reached by 4 hours post exposure. Finally, at 0.5 × MIC of sodium hypochlorite, *E. coli* O157:H7 concentration increased to a final concentration of 8.91 $\log_{10}$(CFU/mL). At 1 × MIC, the bacteria were killed by 1 hour of exposure, and at 2 × MIC, the bacteria were killed by 20 minutes of exposure.

3.3.3 Footbath time-kill assays

As seen in Figure 3.2A, the *E. coli* O157:H7 in footbaths without disinfectant or organic matter decreased in concentration over time, but in footbaths with organic matter, *E. coli* O157:H7 increased in concentration. Without organic matter, the concentration of *E. coli* O157:H7 gradually decreased throughout the course of the trial to a mean concentration of 4.06, 3.53, and 2.50 $\log_{10}$(CFU/mL) at 4 hours for 22˚C, 37˚C, and 42˚C, respectively. At

37˚C and 42˚C, *E. coli* O157:H7 in the organically contaminated simulated footbaths increased in concentration by approximately 1.5 $\log_{10}$(CFU/mL) over 24 hours.

In the organically contaminated footbaths in which Clorox® Bleach was used as a disinfectant, the bacteria were reduced by at least 3 $\log_{10}$(CFU/mL) on contact in the footbaths that were incubated at 22˚C, but bacteria below the limit of detection were still recovered upon enrichment (Figure 3.2B). At 37˚C and 42˚C, *E. coli* O157:H7 concentrations reached a 3 $\log_{10}$(CFU/mL) reduction by 15 and 30 minutes of exposure, respectively, again with bacteria recovered below the limit of detection throughout the trials. By 24 hours of exposure, *E. coli* O157:H7 began to recover and reached 3.37 and 3.58 $\log_{10}$(CFU/mL) at 37˚C and 42˚C, respectively (Figure 3.2B). Bacterial concentrations remained below a three $\log_{10}$(CFU/mL) reduction at 22˚C, yet viable *E. coli* O157:H7 were still recovered at 24 hours.

In the Virex® II 256 footbaths with organic contamination, *E. coli* O157:H7 concentrations were reduced by at least 3 $\log_{10}$(CFU/mL) within 15 minutes of exposure at all temperatures, but viable bacteria were also recovered throughout the experiment for each temperature. As shown in Figure 3.2C, by 24 hours at each temperature, the bacteria had recovered and began replicating. The *E. coli* O157:H7 concentrations reached 3.75 $\log_{10}$(CFU/mL), 5.39 $\log_{10}$(CFU/mL), and 5.19 $\log_{10}$(CFU/mL) at 22˚C, 37˚C, and 42˚C, respectively by 24 hours.

In the organically contaminated footbaths with Virkon™ S, at all temperatures, the *E. coli* O157:H7 concentrations were reduced by at least 3 $\log_{10}$(CFU/mL) on contact (Figure 3.2D). Viable *E. coli* O157:H7 was recovered below the limit of detection at 0 minutes for

the footbaths incubated at 22˚C and 37˚C, and at 15 minutes for the 42˚C footbaths, but no bacteria were recovered at any other time point.

When each disinfectant was added to footbaths without organic contamination, the *E. coli* O157:H7 strains were killed on contact, regardless of the type of disinfectant or the incubation temperature of the footbath (data not shown).

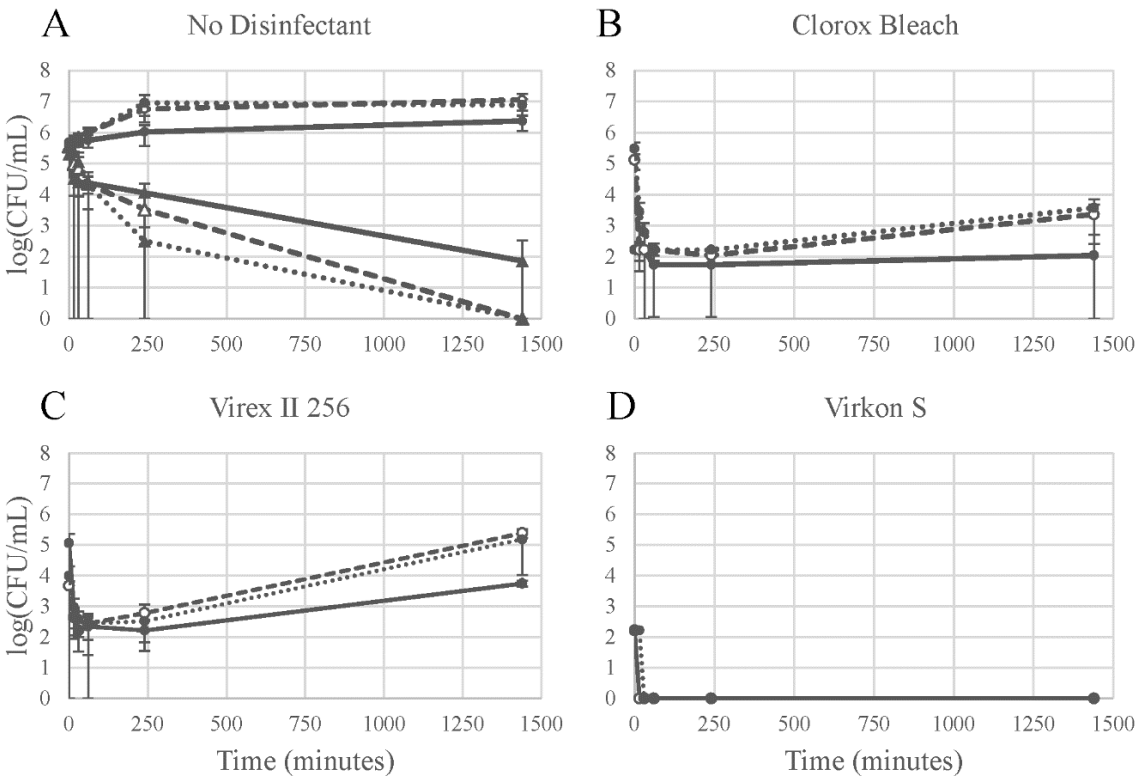

Figure 3.2. Viable *Escherichia coli* O157:H7 concentrations over time in simulated footbaths. Footbaths with no disinfectant (A) or a commercially available disinfectant at the manufacturer's recommended concentration (Clorox® Bleach, B; Virex® II 256, C; Virkon™ S, D) were inoculated with one of three strains of *E. coli* O157:H7. Footbaths were incubated at 22˚C (closed circles with solid lines), 37˚C (open circles with dashed lines), and 42˚C (closed circles with dotted lines). A-D display values for footbaths containing organic matter (circles), while A also displays values for footbaths without organic matter (22˚C, closed triangle with solid line; 37˚C, open triangle with dashed line; 42˚C, closed triangle with dotted line). Concentrations were enumerated at 0 min, 15 min, 30 min, 1 hr, 4 hrs, and 24 hrs, and concentrations for the three strains were averaged.

3.4 Discussion/Conclusions

The primary objective of our study was to evaluate disinfectant efficacy in killing *E. coli* O157:H7 in a footbath environment. Our results support high disinfectant efficacy at killing *E. coli* O157:H7 *in vitro*; however, in a simulated footbath environment with organic fecal contamination, efficacy was variable. The amount of time, the temperature at which the footbath was held, and the specific disinfectant product all appeared to influence the concentration of *E. coli* O157:H7 recovered. These results suggest that *E. coli* O157:H7 may persist in footbaths under certain conditions, causing the footbath to be a potential source of contamination. These conditions can be optimized to decrease the likelihood of *E. coli* O157:H7 persistence within footbaths.

With the exception of phenol, the laboratory-grade disinfectant compounds killed the bacteria within 24 hours at 1 × MIC *in vitro* (Figure 3.1). Every disinfectant compound killed the bacteria within 4 hours of exposure at 2 × MIC. The actual concentration of each disinfectant compound we evaluated is greater than two times the MIC that we report in commercial products.

Didecyl dimethyl ammonium chloride is a quaternary ammonium compound (QAC) that is commonly used in a variety of disinfectants (Melin *et al*., 2014). The QACs are also one of the most common classes of disinfectant compounds used in food processing facilities and clinical environments to control the spread of bacteria (Bloomfield, 2002; Tezel, Pierson, and Paylostathis, 2007). In general, QACs work by irreversibly binding to phospholipids and proteins within the membranes of bacteria, which hinders the permeability of the membrane (Maris, 1995). Many studies have confirmed the efficacy of QACs against *E. coli* O157:H7, though certain types of QACs may be more effective than others. Previous work has shown

that *E. coli* O157:H7 has an MIC_{50} of 2 µg/mL and an MIC_{90} of 4µg/mL when treated with DDAC (Beier *et al.*, 2012). Our study found that the MICs of disinfectants towards *E. coli* O157:H7 differed slightly depending on the media in which the bacteria were growing. According to our results, the MIC for DDAC towards *E. coli* O157:H7 in MHB was 3.2 ppm (3.2 µg/mL), while in M9 minimal media, the MIC was 1.6 ppm (1.6 µg/mL) (Table 3.2). At 0.5 × MIC in MHB, DDAC did not inhibit the growth of *E. coli* O157:H7, but at 1 × and 2 × MIC, DDAC killed the bacteria by 4 hours and 20 minutes, respectively (Figure 3.1A).

Our results indicate that *E. coli* O157:H7 may persist within organically contaminated footbaths filled with the QAC-based disinfectant Virex® II 256. A 3 $\log_{10}$(CFU/mL) reduction was achieved within 15 minutes, but by 24 hours, viable *E. coli* O157:H7 was recovered (Figure 3.2C). Temperature seemed to have a limited, but apparent, effect on *E. coli* O157:H7 concentration in these footbaths, as more *E. coli* O157:H7 was recovered at 37˚C and 42˚C than at 22˚C at the 24 hour time point. Previous studies have found that when boots are dipped in footbaths or footmats containing QAC-based disinfectants, there is little to no decrease in the concentration of bacteria recovered from the sole of the boot when compared to controls (Morley et al., 2005; Hornig et al., 2016). Taking this into consideration with our findings that indicate *E. coli* O157:H7 persistence within footbaths filled with a QAC-based disinfectant, these disinfectants should not be used in footbaths to maximize their efficacy.

Glutaraldehyde is similar to formaldehyde, though it is less toxic, making it more favorable for use in disinfectants. Its mode of action is similar to formaldehyde, in that it works by denaturing proteins and nucleic acids through alkylation (Maris, 1995). Consistent with our findings, glutaraldehyde has previously been shown to be effective at eliminating *E.*

coli O157:H7. A 2% solution of glutaraldehyde reduced *E. coli* O157:H7 by greater than 6.9 $\log_{10}$(CFU/mL) (Sagripanti *et al.*, 1997). We found the MIC of glutaraldehyde toward *E. coli* O157:H7 to be 625 ppm (0.0625%) in MHB, and 5 ppm (0.0005%) in M9 (Table 3.2). The MIC of glutaraldehyde was greatly reduced in M9 versus MHB compared to the other disinfectants. We hypothesize that because MHB contains proteins and nucleic acids within the media, there are additional substances for the glutaraldehyde to bind. This would cause the active concentration of disinfectant available to decrease, reducing bactericidal activity. Proteins and nucleic acids are not present in M9 minimal media, so the concentration with activity against *E. coli* O157:H7 would be higher. We would expect the efficacy of a glutaraldehyde-based disinfectant to be decreased in a footbath contaminated with organic matter, as much of the glutaraldehyde would bind to proteins and amino acids in the organic matter. At 0.5 × MIC of glutaraldehyde in MHB, the *E. coli* O157:H7 initially reduced in concentration for the first 4 hours, but then were able to recover, and the concentration increased over the starting concentration by 24 hours (Figure 3.1B). We suspect that all of the available glutaraldehyde at this concentration was irreversibly bound by organic molecules within a few hours, thus allowing the bacteria to grow without the disinfectant hindering their growth. At 1 × MIC and 2 × MIC, glutaraldehyde rapidly killed the *E. coli* O157:H7, eliminating the organisms within minutes.

Hydrogen peroxide is frequently used as a disinfectant due to its relative non-toxicity. Hydrogen peroxide kills bacteria by oxidizing lipids, proteins, and nucleic acids, with most of the damage due to oxidation of the organism's DNA (Demple, 1991; Hagensee and Moses, 1989; Imlay and Linn, 1988). Previous studies have demonstrated that hydrogen peroxide is capable of reducing pathogen levels by 1 to 6 $\log_{10}$(CFU/mL) (Alvarado-Casillas

et al., 2007; Fouladkhah and Avens, 2010; Materon, 2003; Sapers *et al*., 2001). We have found that the MIC of hydrogen peroxide towards *E. coli* O157:H7 is 40 ppm in MHB and 10 ppm in M9 (Table 3.2). When exposed to 0.5 × MIC and 1 × MIC of hydrogen peroxide, the bacteria were similarly reduced for the first 4 hours, but after 4 hours at 0.5 × MIC, the concentration began to increase (Figure 3.2C). At 1.0 × MIC, the *E. coli* O157:H7 concentration was continually reduced and bactericidal activity was achieved. At 0.5 × MIC, the bacteria may have been able to neutralize all of the hydrogen peroxide in the media after 4 hours of exposure, thus allowing the bacteria to replicate once hydrogen peroxide levels were low. Further work is needed to determine if *E. coli* O157:H7 is able to reduce hydrogen peroxide at varying concentrations, or if other factors are affecting the efficacy of hydrogen peroxide.

When exposed to the peroxygen-based disinfectant Virkon™ S in the organically contaminated footbaths, *E. coli* O157:H7 was killed on contact and did not recover (Figure 3.2D). This disinfectant was the most efficacious at killing and preventing *E. coli* O157:H7 persistence within footbaths, and this observation held true at varying temperatures. Other studies have also reported the effectiveness of peroxygen-based disinfectants. Peroxygen disinfectants consistently rank as the most, or one of the most, effective disinfectants at reducing bacterial concentrations on boot soles as compared to other disinfectants (Hornig *et al.*, 2016; Morley *et al.*, 2005; Dunowska *et al.*, 2006). Bacterial counts on boot soles treated with peroxygen disinfectants in footbaths were 1.3 to 1.4 log lower than untreated boots (Dunowska *et al.*, 2006). Although this does not equate to disinfection, it does show that the peroxygen-based disinfectant is capable of reducing bacterial load on the soles of boots. As

evidenced by our results, peroxygen-based disinfectants such as Virkon™ S are practical disinfectants that can be used to prevent persistence of *E. coli* O157:H7 within footbaths.

Phenol works as a disinfectant by disrupting the cell membrane through inactivation of intracytoplasm enzymes. In low concentrations of phenol, cellular contents are released into the external media, whereas in high concentrations, phenol can inhibit permeases and cause lysis of the cell membrane (Joswick *et al*., 1971; Russell, 1983). When acting against *E. coli* O157:H7, phenol had an MIC of 2500 ppm in MHB, with a two-fold reduction to 1250 in M9 (Table 3.2). Similar to DDAC, at 0.5 × MIC of phenol, the bacteria were still capable of increasing in concentration throughout the duration of the assay (Figure 3.1D). Interestingly, at 1.0 × MIC, the concentration did not change, but remained fairly stable throughout 24 hours of exposure, indicating static activity. At 2 × MIC, the bacteria steadily decreased in concentration until they were killed by 4 hours of exposure. Previous studies have also evaluated the efficacy of commonly-used phenolic disinfectants in footbaths. These disinfectants tend to be ineffective at reducing bacterial load on the bottom of soles. In each study in which phenolic-based disinfectants were used in footbaths, there was no significant decrease or no detectable reduction of bacterial concentration on the soles of boots after stepping through the footbath (Hartmann, Dusick, and Young, 2013; Hornig *et al.*, 2013).

Sodium hypochlorite and other chlorine-based compounds are commonly used as disinfectants in washes and sprays, and are preferred due to their low cost and minimal impact on product quality (Cherry, 1999; Walker and LaGrange, 1991; Carlin and Nguyen, 1994; Herdt and Feng 2009). These disinfectants kill pathogens by oxidizing thiol groups and denaturing proteins (Wang *et al.*, 2012). The presence of organic matter, high temperatures, and light exposure have all been shown to decrease the activity of chlorine-based

disinfectants (Rodgers *et al*., 2003; Shen *et al*., 2012). Our study found that the MIC of sodium hypochlorite towards *E. coli* O157:H7 was 320 ppm in MHB, and was greatly reduced to 1.25 ppm in M9 (Table 3.2). Considering the chlorine released by sodium hypochlorite in solution oxidizes thiol groups in proteins, the disinfectant compound could have been used up by proteins in the MHB media, making less of the disinfectant available to kill the bacteria. This would not occur in the minimal media due to the lack of proteins, so more of the disinfectant would be available for bactericidal activity.

When *E. coli* O157:H7 was exposed to organically contaminated footbaths filled with a Clorox® bleach solution, the *E. coli* O157:H7 concentration reached a 3 $\log_{10}$(CFU/mL) reduction on contact at 22˚C, by 15 minutes at 37˚C, and by 30 minutes at 42˚C, but viable *E. coli* O157:H7 was still recovered from the footbaths throughout the assay (Figure 3.2B). By 24 hours, the *E. coli* O157:H7 reached a concentration of 3.37 and 3.58 $\log_{10}$(CFU/mL) at 37˚C and 42˚C, respectively. Since it took longer for the *E. coli* O157:H7 concentration to reach a 3 $\log_{10}$(CFU/mL) reduction at 42˚C than at 22˚C or 37˚C, we suspect that the increased temperature decreased the activity of the disinfectant. Furthermore, since *E. coli* O157:H7 remained viable in these footbaths, we suspect that the organic matter decreased the efficacy of the disinfectant by deactivating the disinfectant compounds, thus decreasing the concentration of available disinfectant for biocidal activity against *E. coli* O157:H7.

In our series of experiments, footbath environments were simulated in plastic boxes. Although we do not expect this to alter the results of our study, there is a chance that the plastic surfaces could provide an alternative source of binding for possible biofilm formation of *E. coli* O157:H7 or other species that were present in the goat feces that may affect the external validity of our results. These surfaces would not be present in an actual rubber

footbath. Additionally, footbaths and *in vitro* cultures were incubated without the presence of light, which could affect the efficacy of the disinfectants. Another consideration in footbath environments at agritourism events would be the amount of organic matter present in the footbaths. In a footbath at this setting, the amount of organic contamination will vary, thus altering the efficacy and level of deactivation of the disinfectant in the footbath.

This study and others have demonstrated that, while footbaths may decrease viable bacterial concentrations, they should not be used as a sole prevention method for complete elimination of potential pathogens (Morley et al., 2005; Dunowska et al., 2006; Hornig et al., 2016; Hartmann, Dusick, and Young, 2013). *Escherichia coli* O157:H7 was shown to have persisted in Clorox® Bleach- and Virex® II 256-filled footbaths that were contaminated with organic matter after 24 hours. Of the three disinfectants examined in this study, Virkon™ S was the most effective at preventing E. coli O157:H7 persistence within footbaths. If footbaths are to be used as a biosafety measure, Virkon™ S or similar peroxygen-based disinfectants would be the most appropriate of those chemicals we evaluated for use. Footbaths should also be cleaned on a regular occasion to remove organic matter. If chlorine- or QAC-based disinfectants are used in the footbath, the footbath should be cleaned as soon as it is apparent that organic matter is accumulating to prevent the persistence of E. coli O157:H7 or other pathogens within the footbath, thus making the use of these products logistically challenging. If Virkon™ S or a similar peroxygen-based disinfectant is used, it may be acceptable to clean the footbath every 24 hours as usually recommended to retain efficacy.

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APPENDICES

Appendix A

Statistical Significance of *Escherichia coli* Biofilm Values

Table A.1. Statistical significance of biomass (OD_{550}) and log_{10}(CFU/mL) values of *Escherichia coli* O157:H7 biofilms grown on polystyrene plastic. Strains with the same letter are not significantly different ($p > 0.05$).

Time	Temperature	Strain	OD_{550}	Strain	log_{10}(CFU/mL)
24	22	ATCC 25922	a	ATCC 25922	a
		217C	a	171F	a, b
		202H	a	202H	b, c
		171F	a	208H	b, c, d
		226F	a	229F	c, d, e
		208H	a	230C	c, d, e
		230C	a	217C	d, e
		229F	a	226F	e
	37	ATCC 25922	a	ATCC 25922	a
		208H	a	202H	b
		202H	a	171F	b
		217C	a	208H	b, c
		171F	a	226F	c, d
		229F	a	217C	d
		226F	a	229F	d
		230C	a	230C	d
	42	ATCC 25922	a	ATCC 25922	a
		208H	a	208H	b
		230C	a	171F	b, c
		217C	a	202H	c
		202H	a	217C	c
		171F	a	229F	c
		226F	a	230C	c
		229F	a	226F	c
48	22	ATCC 25922	a	ATCC 25922	a
		171F	b	171F	b
		202H	c	202H	c
		208H	c	208H	c
		229F	c	230C	c, d
		226F	c	229F	c, d
		217C	c	226F	c, d
		230C	c	217C	d
	37	ATCC 25922	a	ATCC 25922	a
		202H	b	171F	b
		229F	b	202H	b
		208H	b	208H	b

Table A.1 (Continued). Statistical significance of biomass (OD_{550}) and log_{10}(CFU/mL) values of *Escherichia coli* O157:H7 biofilms grown on polystyrene plastic. Strains with the same letter are not significantly different ($p > 0.05$).

Time	Temperature	Strain	OD_{550}	Strain	log_{10}(CFU/mL)
48	37	171F	b	230C	b, c
		230C	b	229F	c
		217C	b	217C	c
		226F	b	226F	d
	42	ATCC 25922	a	ATCC 25922	a
		208H	a	171F	b
		171F	a	202H	b
		202H	a	208H	b, c
		217C	a	230C	c, d
		229F	a	229F	d
		226F	a	217C	d, e
		230C	a	226F	e
72	22	ATCC 25922	a	ATCC 25922	a
		171F	b	171F	a, b
		208H	c	208H	b, c
		229F	c	229F	b, c
		202H	c, d	202H	c
		226F	c, d	230C	d
		230C	d	226F	d
		217C	d	217C	d
	37	ATCC 25922	a	ATCC 25922	a
		208H	b	171F	b
		171F	b, c	208H	b
		202H	b, c	202H	b
		226F	c	230C	c
		217C	c	217C	c, d
		230C	c	229F	c, d
		229F	c	226F	d
	42	ATCC 25922	a	ATCC 25922	a
		208H	a, b	208H	a
		171F	a, b	171F	a, b
		202H	a, b	202H	b
		226F	a, b	217C	c
		229F	a, b	230C	c
		217C	a, b	229F	c
		230C	b	226F	c

Table A.2. Statistical significance of biomass (OD_{550}) and log_{10}(CFU/mL) values of *Escherichia coli* O157:H7 biofilms grown on rubber footbaths. Strains with the same letter are not significantly different ($p > 0.05$).

Temperature	Strain	OD_{550}	Strain	log_{10}(CFU/mL)
22	202H	a	230C	a
	171F	a, b	217C	a
	208H	a, b	171F	a, b
	230C	a, b	ATCC 25922	b, c
	ATCC 25922	a, b	208H	b, c
	217C	a, b	229F	b, c
	226F	a, b	226F	c
	229F	b	202H	c
37	ATCC 25922	a	ATCC 25922	a
	217C	a	171F	a, b
	226F	a	230C	a, b
	208H	a	217C	a, b
	171F	a	208H	b
	202H	a	202H	c
	230C	a	229F	c
	229F	a	226F	c
42	ATCC 25922	a	ATCC 25922	a
	229F	a	217C	b
	171F	a	230C	b, c
	202H	a	171F	b, c
	226F	a	208H	b, c, d
	208H	a	229F	c, d, e
	230C	a	202H	d, e
	217C	a	226F	e

Appendix B

Confocal Microscopy of *Escherichia coli* O157:H7 Biofilms

Confocal microscopy of *E. coli* O157:H7 biofilms

Seven *E. coli* O157:H7 strains were independently inoculated into 2 mL of lysogeny broth (LB; Sigma-Aldrich, St. Louis, MO) and incubated overnight at 37°C. The cultures were adjusted to a density of 1 McFarland using a DensiCHEK plus (BioMerieux). Five-hundred microliters of M9 minimal media was added to each well in glass chamber slides (Lab-Tek, Thermo Fisher Scientific, Rochester, NY). The chambers were inoculated with 50 µL of the concentration adjusted *E. coli* O157:H7 cultures, and the slides were incubated for 72 hours at 22°C with shaking.

Following incubation, the media was gently removed from each chamber, and 750 µL of phosphate buffered saline (PBS; Fisher BioReagents, Fair Lawn, NJ) was added to each chamber to remove loosely adhered planktonic cells. The PBS was removed, and the wash was repeated once. Five-hundred microliters of PBS was added to each chamber, and 25 µL of 10× SYTO 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, Thermo Fisher Scientific, Eugene, OR) and 50 µL of 10× Wheat Germ Agglutinin, Alexa Fluor 350 Conjugate (WGA; Invitrogen, Thermo Fisher Scientific, Eugene, OR) were added to the chambers. The slides were incubated for 15 minutes with shaking at 37°C in the dark. The solution was removed, and 500 µL of SYPRO Ruby biofilm matrix stain (Invitrogen, Thermo Fisher) was added to each chamber. The slides were again incubated for 15 minutes with shaking at 37°C in the dark. The solution was removed, and 250 µL of PBS with 250 µL of 4% paraformaldehyde was added to each chamber. The slides were incubated at 4°C for 15 minutes in the dark. The solution was removed, and 500 µL of PBS was added to each chamber. The stained slides

were stored at 4°C in the dark until viewing. The PBS and chambers were removed from the slides, and glass coverslips were placed over each sample. The samples were viewed with a Zeiss LSM 710 laser scanning confocal microscope (Cellular and Molecular Imaging Facility, North Carolina State University). Biofilm growth was observed for five of the seven strains (202H, 217C, 226F, 229F, and 230C; Figure B). Interestingly, *E. coli* ATCC 25922 did not appear to form dense biofilms, although an abundance of planktonic cells were observed. This in in contrast to our other data that suggests strains ATCC 25922, 171F, 202H, 208H, and 229F formed biofilms under these incubation conditions. However, these biofilms were formed on polystyrene plastic, so differences in surface material may affect their potential to form biofilm. The SYTO 9 nucleic acid stain was detected in every strain, regardless of biofilm formation. In the strains with apparent biofilm formation, more of the WGA stain was detected, indicating a greater amount of polysaccharides (Figure B).

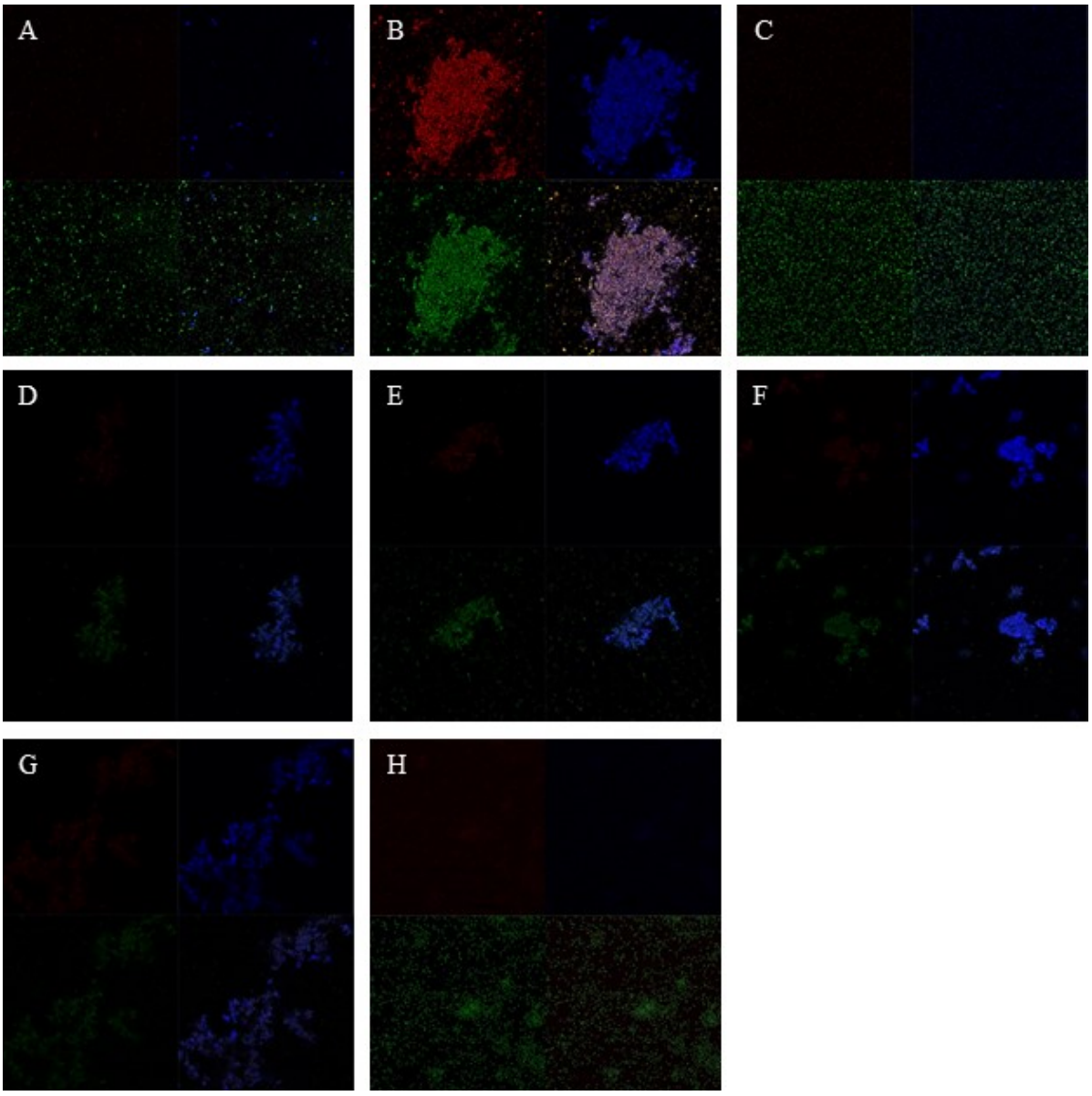

Figure B. Confocal laser scanning micrographs of *Escherichia coli* biofilms on a glass slide. Samples were stained with SYPRO (proteins; top left), WGA (polysaccharides; top right), and SYTO 9 (nucleic acids; bottom left). The bottom right is an overlay of all three stains. The strains include the *E. coli* O157:H7 strains 171F (A), 202H (B), 208H (C), 217C (D), 226F (E), 229F (F), and 230C (G), and the control strain *E. coli* ATCC 25922 (H).

Appendix C

Polystyrene Plastic Pegs and Rubber Footbath Pieces Used for Biofilm Assays

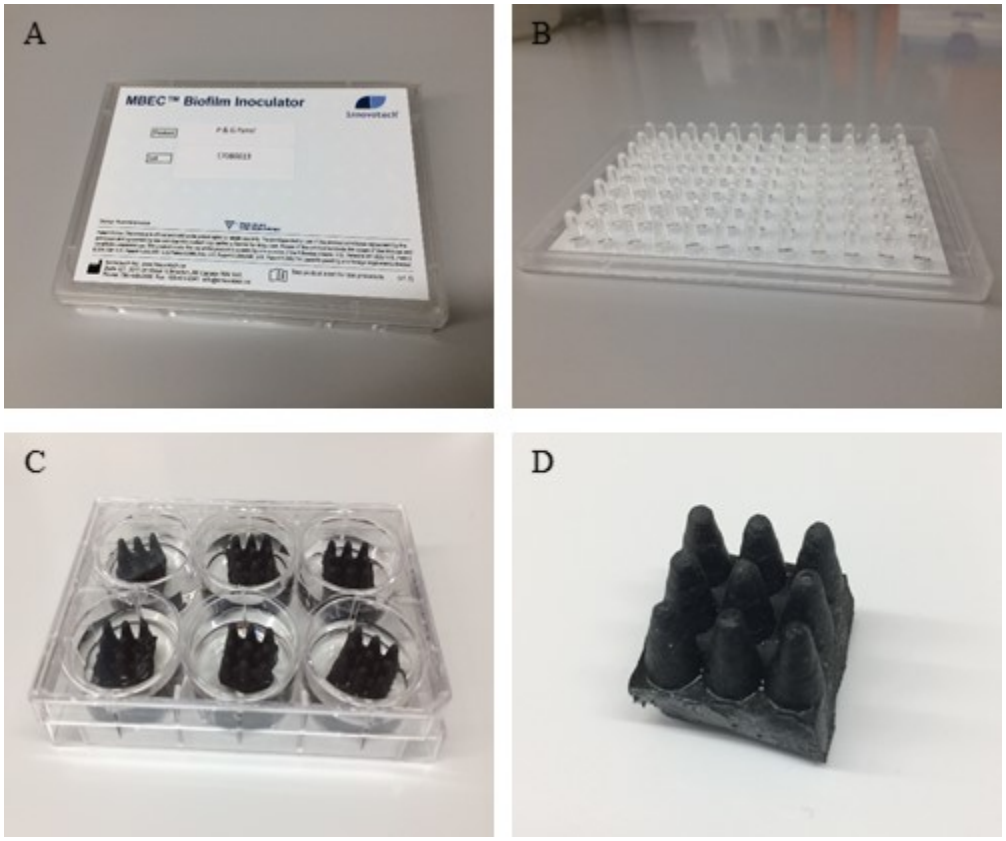

Figure C. Polystyrene plastic pegs and rubber footbaths pieces on which the biofilm-forming potential of *Escherichia coli* O157:H7 strains were determined. The *E. coli* O157:H7 strains and the biofilm positive control strain *E. coli* ATCC 25922 were inoculated in M9 minimal media within 96-well plates (A) and 6-well plates (C) to determine their biofilm-forming potential on polystyrene plastic pegs (A and B) and rubber footbath pieces (D), respectively.

Appendix D

Growth Curves of *Escherichia coli* O157:H7

Growth of *E. coli* O157:H7 in rich media versus minimal media

Eighteen hours before the beginning of the assay, *E. coli* O157:H7 strain 202H was inoculated in 2 mL of M9 minimal media (Sigma-Aldrich, St. Louis, MO) and 2 mL of Mueller-Hinton Broth (MHB; Thermo Scientific). Both cultures were incubated at 37°C for three hours, and their concentrations were adjusted to 0.5 McFarland using a DensiCHEK plus (BioMerieux). The cultures were diluted 1:100 by adding 50 µL of the enrichment to 5 mL of their respective media. These cultures were incubated at 37°C for 12 hours and were used to determine bacterial concentrations after 12 hours of growth.

A second set of M9 and MHB cultures were prepared similarly at the beginning of the assay, and these cultures were used to determine bacterial concentrations at 0 to 12 hours of growth. A 100 µL aliquot of from each set of cultures was removed every hour for 12 hours, serially diluted ten-fold, and spotted in triplicate on tryptic soy agar (TSA; Becton Dickinson, Sparks, MD). The TSA plates were incubated at 37°C overnight, and colonies were counted to determine the CFUs/mL at each time point.

The *E. coli* O157:H7 concentration increased more rapidly in MHB than in M9 minimal media (Figure D). In MHB, the bacteria reached stationary phase approximately 6 hours after inoculation, whereas stationary phase was not reached until approximately 13 hours after inoculation in M9 minimal media. The final bacterial concentration in MHB (9.1 $\log_{10}$(CFU/mL)) was also slightly higher than the final bacterial concentration in M9 (8.9 $\log_{10}$(CFU/mL)). These differences are likely due to nutrient limitation in M9 minimal media versus MHB, and may provide evidence as to why the MICs of several disinfectants towards

E. coli O157:H7 are lower in M9 versus MHB. If the bacteria are stressed from lack of nutrients, they may be more susceptible to disinfectants than if nutrients are readily available. Additionally, certain disinfectants may be bound up by the nutrients available in rich media, such as proteins and amino acids, causing reduced active concentrations of the disinfectant and increased survivability of the bacteria.

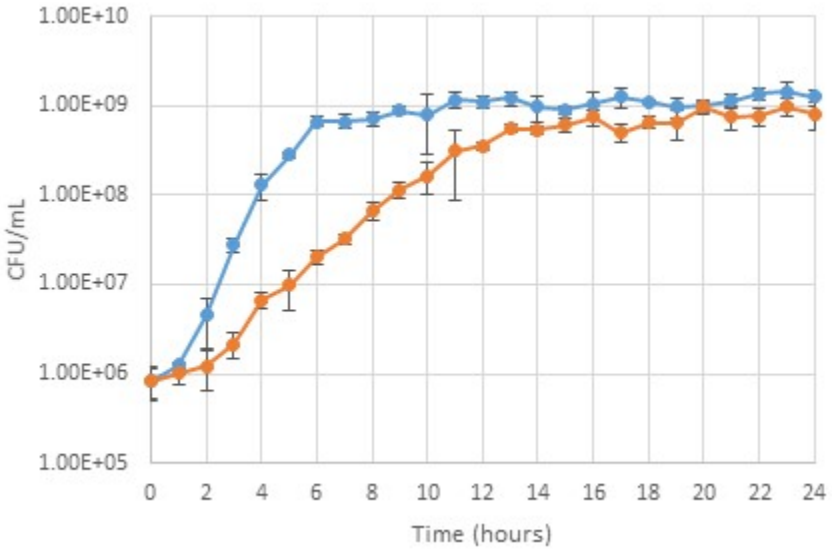

Figure D. Concentration of *Escherichia coli* O157:H7 strain 202H recovered from a rich (Mueller Hinton Broth; blue) and a minimal (M9; orange) media at 37°C.