RELATIONSHIP BETWEEN DESICCATION TOLERANCE AND BIOFILM FORMATION IN SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) is a major concern in the food industry and requires effective control measures to prevent foodborne illnesses. Previous studies have demonstrated increased difficulty in the control of biofilm-forming STEC. Desiccation, achieved through osmotic stress and water removal, has emerged as a potential antimicrobial hurdle. This study focused on 254 genetically diverse *E. coli* strains collected from cattle, carcass hides, hide-off carcasses, and processing equipment. Of these, 141 (55.5%) were STEC and 113 (44.4%) were generic *E. coli*. The biofilm-forming capabilities of these isolates were assessed, and their desiccation tolerance was investigated to understand the relationships between growth temperature, relative humidity (RH), and bacterial survival. Only 28% of the STEC isolates had the ability to form biofilms, compared to 60% of generic *E. coli*. Stainless steel surfaces were exposed to different combinations of temperature (0°C or 35°C) and RH (75% or 100%), and survival rates were measured over 72 h and compared to controls. The results revealed that all the strains exposed to 75% RH at any temperature had reduced growth ($p < 0.001$). In contrast, 35°C and 100% RH supported bacterial proliferation, except for isolates forming the strongest biofilms. The ability of *E. coli* to form a biofilm did not impact growth reduction at 75% RH. Therefore, desiccation to 75% RH at temperatures of 0°C or 35°C holds promise as a novel antimicrobial hurdle for the removal of biofilm-forming *E. coli* from challenging-to-clean surfaces and equipment within food processing facilities.

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LIST OF ABBREVIATIONS

WHO	World Health Organization
STEC	Shiga Toxin Producing *E. Coli*
HUS	Hemolytic Uremic Syndrome
aw	Water Activity
ETEC	Enterotoxigenic *E. coli*
EPEC	Enteropathogenic *E. coli*
EIEC	Enteroinvasive *E. coli*
DAEC	Diffusely Adherent *E. coli*
EAEC	Enteroaggregative *E. coli*
EUR	European Region
WPR	Western Pacific Region
RAJ	Recto Anal Junction
EPS	Extracellular Polymeric Substance
AHL	Acylated Homoserine Lactones
QS	Quorum Sensing
AIPs	Auto-Inducing Peptides
IPs	Lipopolysaccharides
AIs	Autoinducers
SS	Super Shedding
HEPs	High Event Periods
HHP	High Hydrostatic Pressure
PEF	Pulse Electric Field
UV	Ultraviolet
PL	Pulsed Light
RTE	Ready to Eat
RBB	Relative Biofilm Biomass
RH	Relative Humidity
LMG	Lactose Monensin Glucuronate Agar

CHAPTER 1

Literature Review

1.1 Introduction

Food products can be contaminated at many points throughout their production chain, including at manufacturing, distribution, preparation and/or final consumption (Hemalata & Virupakshaiah, 2016). The chances of foodborne contamination largely depends on workers, handling, processing, knowledge, practice of sanitation and food hygiene practices (Aklilu et al., 2015). According to World Health Organization (WHO), foodborne illness can be defined as disease caused by consuming contaminated food and water (Kadariya et al., 2014). There are three types of foodborne infection. Intoxication (a toxin produced by the pathogens causes food poisoning), infection (ingestion of viable pathogens), and toxicoinfection (organism produces toxins while living in the gastrointestinal tract) (Desta Sisay, 2015; Dhama et al., 2013). Foodborne infections can cause severe harm and death in immunocompromised, aged, and young people (Bintsis, 2017).

Foodborne pathogens are microorganisms which include bacteria, viruses, fungi, and some eukaryotic parasites (Zhao et al., 2014). Among these, bacteria are a major cause of foodborne illness in humans. The *Enterobacteriaceae* family is a substantial threat to the food industry. When ingested, pathogenic bacteria from this family can cause enteric disease (Kolling et al., 2012). There are several pathogens from the enteric family, but *Salmonella* and *E. coli* are the most serious threats to the food industry (Matthew Wells, 2021).

Among the foodborne illness related to meat, STEC is a major concern for public health because of worldwide reported outbreaks and sporadic cases (Majowicz et al., 2014). Hussein (2007) highlights that the presence of STEC in Canadian and American beef industries is intermittent. The presence of antimicrobial barriers is essential in slaughter plants to prevent STEC contamination (Liu et al., 2021). Contamination can happen if these barriers fail

(Hussein, 2007). Biofilm formation by STEC can lead to contamination, highlighting the need to comprehend the factors that contribute to antimicrobial measures failing (Bosilevac & Koohmaraie, 2011). Detached biofilms can continuously contaminate slaughter plants and are harder to sanitize compared to planktonic cells, emphasizing the need to explore biofilm formation and sanitizer tolerance in STEC (Bosilevac & Koohmaraie, 2011).

Effective antimicrobial hurdles in food processing facilities resulted in declining STEC O157:H7 infections in Canada from 2000 to 2010 (Pollari et al., 2017). However, in 2010 a worldwide study showed an estimated 31 foodborne hazards that caused an estimated 600 million foodborne illnesses and approximately 420,000 deaths, producing 33 million disability-adjusted life years (Havelaar et al., 2015). In 2012, there was a noteworthy outbreak in Canada due to *E. coli* O157:H7 in beef and there were 18 reported illness, and millions of dollars of beef recalls, and a comprehensive examination of the impacted meat products was immediately started (Lewis et al., 2013). In 2018, there were 7 and 9 recall outbreaks in Canada and United States, respectively, related to beef products (Canadian Food Inspection Agency 2019). According to latest available statistics, 2 recalls due to STEC and 13 related to beef occurred out of 47 total food-related recalls in the United States (United States Department of Agriculture, Recall Summaries 2021). Recall incidents in the U.S. concerning STEC and beef prior to 2014 have caused Canadian producers to face economic losses between 8 and 67 million dollars (Capps et al., 2013). United States recalls also have an impact on Canadian beef prices (Cranfield, 2013). In 2023 a major *E. coli* outbreak in daycares across the Calgary area in Alberta, Canada, led to 39 hospitalized individuals, including 38 children. Hemolytic uremic syndrome (HUS) was diagnosed in 23 of the hospitalized cases, with 8 patients undergoing peritoneal dialysis (Freedman et al., 2024; Heidenreich, 2023; Rodriguez, 2023).

Cattle are the main reservoir of STEC, and approximately 150 different STEC serotypes have been linked to human disease. Whereas other serotypes are emerging, outbreaks and sporadic STEC infections have been predominantly connected to O157:H7 (Møretrø et al., 2010). STEC have various virulence factors, including Shiga toxins (*stx*), intimin (*eae*) and enterohemolysins (*ehxA*) (Law, 2000). The STEC are mostly asymptomatic in cattle but can produce severe diarrhea in young calves (Johnson et al., 1996). The symptom spectrum in humans includes mild to bloody diarrhea, which is frequently accompanied by severe abdominal cramps and in more severe cases, HUS (Bell & Kyriakides, 1998; R. P. Johnson et al., 1996).

Pathogenic bacteria attach to different equipment surfaces and may form biofilm (Moser et al., 2021). This is the most common microbial form with over 90% of bacteria existing in biofilms which forms on various biotic and abiotic surfaces, including stainless steel, rubber, plastic, silicon and glass in food manufacturing settings (Ribeiro et al., 2019). Development of biofilms by STEC is thought to be a major reason for outbreaks (Winfield & Groisman, 2003). Biofilm is defined as a sessile structure of microorganisms in which they are embed and protect themselves from external factors and substances to give extreme resistance against sanitizers, oxidizing agents, antibiotics, desiccation and high/low temperature (Wang et al., 2016). Biofilms could develop on food processing equipments after exposure to biofilm-forming bacteria (Ryu et al., 1999). This sequential process starts from the attachment, microcolony formation, and then the formation of a mature biofilm. The sporadic cross-contamination of biofilm producing STEC can contaminate beef (Stoodley et al., 1998).

Studies indicate that, about 95% of O157:H7 isolates lack significant biofilm-forming capabilities (Uhlich et al., 2013). Biofilm formation is more common in non-O157 *E. coli* isolates than in O157:H7 isolates (Cookson et al., 2002; Stanford et al., 2021). Biofilm

formation in STEC isolates is hindered by a *stx1* prophage inserted in *mlrA*, inhibiting curli fimbriae expression, a mutation in *rpoS* resulting in decreased cellulose and curli expression, and impaired motility impacting curli expression and initial biofilm attachment (Chen et al., 2013).

Meat processors have taken proactive steps to prevent contamination by implementing enhanced cleaning protocols that target biofilms, underscoring the significance of proactive measures for food safety (Foods, 2023). However, there is a lack of scientific data on how STEC biofilms contribute to beef contamination, highlighting the need for more research in this field (Canadian Food Inspection Agency (2019b).

Both physical and chemical methods have been studied in the food manufacturing environment to prevent bacterial biofilm formation. Previous studies demonstrated that mechanical treatments such as clean-in-place are not eliminated all the bacterial cells. In contrast, chemical treatments have some potential to control biofilm formation and kill bacterial cells (Yu et al., 2021). There are various previously described methods to control biofilm, including chemical, physical, and mechanical methods, such as essential oils (Burt, 2004), enzymes (Maszewska et al., 2021; Seghal Kiran et al., 2014), biosurfactants (Salisbury et al., 2021), photosensitization (Yu et al., 2021), ultrasonic waves (Bigelow et al., 2008; Oulahal-Lagsir et al., 2000; Yu et al., 2020), and electric fields (Ravikumar et al., 2019; Sabelnikov et al., 1991).

Salt is commonly used with other technologies in the food industry as a preservative and antibacterial agent (Desmond, 2006). Salt can cause damage to bacterial cells by disrupting the osmotic balance between the cytoplasm and intracellular environment (Csonka, 1989). This osmotic dehydration process can be used for partial dewatering by immersion in hypertonic solutions (Rahman, 2007). Water efflux occurs when bacterial cells are exposed to

a low water activity (aw) compared to the cellular component. A short exposure with a significant difference between aw can causes the shrinkage of the cytoplasm (Potts, 1994). The removal of water from cells by applying osmotic stress is termed desiccation. It can be obtained either through slow or rapid desiccation (Potts, 1994).

Several research studies reported that *E. coli* O157:H7 could survive on stainless steel surfaces for prolonged periods (26-80 days) at low temperatures (4°C and 18°C) (Maule, 2000; Wilks et al., 2005). This demonstrates that STEC can survive at various surface temperatures and cause cross-contamination from surfaces to food items.

In practice, *E. coli* encounters various environmental factors, including low/high temperature, stress environment such as pH variations, osmotic stress, antimicrobial exposure, nutrient availability, survival time and attachment to surfaces.

1.2 STEC History & Classification

The first general recognition of *E. coli* O157:H7 as an enteric pathogen came in 1982 after an outbreak in Oregon, USA, where 47 individuals fell ill after consuming hamburgers from a McDonald's restaurant (The Marler Clark Network., 2020). Subsequently, the devastating Jack-in-the-Box outbreak in 1993 became one of America's most tragic foodborne incidents. It resulted in the loss of four lives and impacted 700 others (The Marler Clark Network, 2008). Historical evidence suggests that illness due to O157 was first documented in 1975 when it was associated with a case of bloody diarrhea (Law, 2000).

Researchers identified significant traits associated with various serotypes as the scientific community delved deeper into the virulence factors of pathogenic *E. coli*. Shiga toxins from *E. coli* O26 and O157 were discovered to have the ability to kill Vero and HeLa cells, showing their potential as pathogens (O'Brien et al., 1983). Moreover, STEC

demonstrated the ability to invade the intestinal epithelium and produce a heat-stable enterotoxin, characteristics not previously associated with *E. coli* strains (O'Brien et al., 1983). It became increasingly clear that *E. coli* was not a monolithic entity but a diverse group of bacteria with distinct pathogenic profiles.

While *E. coli* O157:H7 garnered significant attention due to its association with severe outbreaks, it was not the sole STEC serovar causing human illness. The diversity within the STEC group was highlighted by earlier incidents involving serovars like O26 and O111 (Ogura et al., 2017). As a result, regulatory agencies have identified a specific group of STEC serovars, called the "Big Six," as a significant public health concern that is closely monitored and reported (Alharbi et al., 2022).

The classification of pathogenic *E. coli* strains underwent refinement as researchers sought to categorize them based on their biological characteristics and pathogenesis. This led to the recognition of six distinct groups, including Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Table 1) (Kaper & O'Brien, 2014). STEC are identified by somatic (O) antigen, featuring 200+ serogroups that can produce Shiga toxins. To assess the pathogenic potential of STEC strains, a new classification scheme known as seropathotypes was proposed, considering virulence, serological, and genetic features (Boerlin et al., 1999; Frankel et al., 1998; Karmali et al., 2003; Nataro & Kaper, 1998).

Table 1: Different types of *E. coli* and their defining characteristics.

Pathotype	Characteristics
Shiga toxin *E. coli* (STEC)	Invades intestinal epithelium. Can form attaching and effacing (A/E) lesions. Produces Shiga toxins. Symptoms of infection can range from moderate to severe. Might lead to the development of HUS. It causes the deterioration of the intestinal membrane. (Law, 2000; O'Brien et al., 1983)
Enterohaemorrhagic *E. coli* (EHEC)	Causes haemorrhagic colitis. Associated with severe colonic and renal diseases. (Goldwater & Bettelheim, 2012) Creates attaching and effacing (A/E) lesions. Destroys intestinal microvilli and integrates into host cytoskeleton.
Enteropathogenic *E. coli* (EPEC)	Creates attaching and effacing (A/E) lesions. Destroys intestinal microvilli and integrates into host cytoskeleton. Main symptom is diarrhea. (Trabulsi et al., 2002)
Enteroaggregative *E. coli* (EAEC)	Does not form attaching and effacing (A/E) lesions. It forms a mucus-like biofilm in the intestinal tract. Various strains are likely to cause diarrhea. Main symptom is diarrhea. (Nataro & Kaper, 1998) Secretes either heat stable (ST) or heat-labile (LT) toxins.
Enterotoxigenic *E. coli* (ETEC)	Either heat-stable or heat-labile toxins or both toxins can be secreted. Illness occurs when ETEC adheres to the intestinal mucosa and begins secreting toxins. (Nataro & Kaper, 1998)
Enteroinvasive *E. coli* (EIEC)	Genetically and biochemically related to *Shigella* spp. Secretes enterotoxin causing diarrhea. (Nataro & Kaper, 1998) Characterized by a random distribution of bacteria along a cell surface.
Diffusely Adherent *E. coli* (DAEC)	Does not show microcolony formation within the intestine. Commonly causes diarrhea by the production of toxins or intestinal inflammation. (Servin, 2014)

The seropathotype model aimed to categorize STEC strains according to their ability to cause sickness and outbreaks. Strains belonging to seropathotype A were more likely to cause HUS and were associated with outbreaks (Karmali et al., 2003). However, this classification's efficacy was questioned following significant outbreaks involving strains not fitting into the

predefined seropathotypes (Messens et al., 2015). The rise in non-O157 STEC cases further highlighted the need for a comprehensive classification scheme that accurately reflects serotype distribution and severity in causing illness (National Enteric Disease Surveillance, 2013).

1.3 Global Outbreaks and Clinical Isolates

Since 1982, there have been severe outbreaks in the United States caused by STEC O157:H7, resulting in hospitalizations, cases of HUS, and fatalities (Kim et al., 2020). Foodborne diseases primarily spread through fecal-oral transmission, but there is also a growing trend of contact with animals (Rangel et al., 2005). In 1990, the initial outbreak of non-O157 STEC, specifically STEC O111, was documented in the USA. Like O157, non-O157 outbreaks often involve food as a primary transmission route (43% foodborne cases for non-O157 and 52% for O157) during 1982–2002 in USA (Luna-Gierke et al., 2014).

STEC infections significantly impact global public health, causing over 1 million illnesses and 100 deaths in 2010, according to the WHO (Havelaar et al., 2015). These infections can cause various symptoms and have the potential to result in severe complications like HUS, posing a significant concern for healthcare systems globally. From 1998 to 2016, 211 STEC outbreaks were reported in the European region (EUR) and Western Pacific region (WPR), with 176 outbreaks in EUR and 35 in WPR (Organization, 2019). While not as common as in the America, these outbreaks emphasize the global presence of STEC-related diseases and the necessity of monitoring and controlling them (Kim et al., 2020).

In 1996, a notable outbreak of O157 STEC took place in Japan with 12,680 reported symptomatic patients, 121 individuals (0.95%) developed HUS, and three deaths (Fukushima et al., 1999). There was a significant rise in STEC cases after the outbreak in Japan, with over

3,000 cases reported between 1999 and 2012. This starkly contrasts the average of only 105 cases per year in 1991-1995 (Terajima et al., 2014). This escalation underscores the wide-ranging impact of STEC outbreaks and highlights the crucial need for robust surveillance and response measures.

Along with O157, various serogroups of STEC, including O26, O111, O103, O121, and O145, have commonly been found to cause infection (Terajima et al., 2014). The rise of non-O157 STEC strains has presented significant obstacles, including a severe outbreak in Germany in 2011. For three months, 3,816 cases of non-O157 STEC (O104) were reported, making it a historic epidemic due to the high incidence of HUS (22.4%, n = 845) and fatalities (Frank et al., 2011). This occurrence emphasizes the dynamic nature of STEC infections, highlighting the necessity for ongoing surveillance and response measures to address new challenges effectively.

According to surveillance reports, the prevalence of STEC infections in Europe is a cause for concern (Kim et al., 2020). The burden of STEC infections significantly increased from 3,573 cases in 2009 (EU, 2011) to 6,073 cases in 2017 (EU, 2018). Despite O157 being the most frequently reported serogroup, the proportion of non-O157 infections grew between 2011 and 2017, demonstrating the ever-changing nature of STEC epidemiology. Germany and the United Kingdom had the highest human STEC infection rates among the 31 European countries, emphasizing the importance of focused interventions and increased surveillance (Kim et al., 2020). These findings highlight the worldwide impact of STEC infections and stress the need for ongoing research and collaboration to reduce the disease burden effectively.

1.4 Role of Animals in STEC Transmission

STEC strains carrying *stx* genes are frequently found in the gastrointestinal tracts of

different animals, but their presence in animals usually has no clinical significance for either animals or humans (Persad & LeJeune, 2014). In humans, disease outcomes are often linked to other virulence factors in addition to the *stx* gene, but animals can act as reservoirs for STEC strains that harm humans or as hosts that aid transmission (Persad & LeJeune, 2014).

The primary reservoir for STEC, especially the O157 serogroup, is cattle, which acquire these bacteria from contaminated food, water, or contact with infected feces (Friedrich et al., 2002; Gyles, 2007). Despite lacking vascular receptors for *stx*, cattle can carry and intermittently excrete STEC, primarily at the recto-anal junction (RAJ) (Naylor et al., 2003; Pruimboom-Brees et al., 2000). Other ruminants like sheep, goats, and deer may also act as reservoirs for STEC. Conversely, birds, swine, dogs, and horses can act as spillover hosts, vulnerable to colonization but incapable of sustaining colonization without exposure to the pathogen (Gyles, 2007).

Various factors, including exposure frequency, host susceptibility, shedding duration, and environmental conditions, make it complex to comprehend the prevalence and dynamics of STEC in animals (Persad & LeJeune, 2014). Farm management practices, such as water sources and animal housing, significantly impact STEC incidence (Fairbrother & Nadeau, 2006; Gagliardi & Karns, 2000; LeJeune, Besser, & Hancock, 2001). Furthermore, the risk of STEC transmission increases due to animal movement, such as transportation for exhibitions (Cernicchiaro et al., 2009).

Most cattle farms in the United States have STEC O157 in the environment, primarily shed intermittently by animals, mainly through feces (Hancock, 2001). Milk from cows with mastitis can also potentially contain STEC (Lira et al., 2004). The occurrence of STEC in cattle populations shows a wide range, from 0 to 71%, with unpredictable fluctuations (Cerqueira et al., 1999). Animals that excrete high concentrations of STEC contribute

significantly to environmental contamination, especially in warmer months (Matthews et al., 2006; Omisakin et al., 2003). Calves, especially following weaning, demonstrate elevated shedding levels, with observed seasonal variations (Nielsen et al., 2002).

1.5 Biofilm Formation and Genetics

Our understanding of microbial communities has evolved from focusing on individual species to comprehending the complex dynamics of diverse microbial communities (Røder et al., 2015; Williams, 2007). In the past, microbiological research centered on studying microorganisms in isolated conditions, overlooking that microorganisms coexist in various environments (Frey-Klett et al., 2011). Recently, there has been a shift towards studying microorganisms in Polymicrobial communities, leading to increased biofilm and intercellular communication research. This change emphasizes the need to investigate the metabolic properties of multi-species systems (Abram, 2015; McNeilly et al., 2021; Yang et al., 2021).

Biofilms are complex structures of bacteria, attached to surfaces, and enveloped in an extracellular polymeric substance (EPS) matrix (Donlan, 2002; Oliveira et al., 2015). The EPS layer makes biofilms more resilient to stress and antimicrobial treatments (Chitlapilly Dass & Wang, 2022; Logan et al., 2018). Understanding biofilm formation becomes more complex because microorganisms vary in their roles within the biofilm (Federle & Bassler, 2003; Hughes & Sperandio, 2008; Oliveira et al., 2015). The synthesis of specific acylated homoserine lactones (AHL) autoinducers is carried out by LuxI-like proteins in gram-negative bacteria (Engebrecht et al., 1983). AHLs are specific signaling molecules present in different species of gram-negative bacteria and are only detected and responded to by bacteria of the same species (Engebrecht et al., 1983). Based on a previous study, AHLs promote an increase in EPS production and the attachment of bacterial cells in *E. coli* (A. & V., 2016). In contrast

to gram-negative bacteria, AHL-mediated quorum sensing (QS) is not utilized by gram-positive bacteria. In contrast, gram-positive bacteria secrete oligopeptide autoinducers, or auto-inducing peptides (AIPs), into their environment. Typically, AIPs are composed of 5 to 17 amino acids and can have unique side chain modifications (Lazazzera & Grossman, 1998). When AIPs reach a certain level they attach to the extracellular segment, causing kinase activation. Following this, the activation initiates the phosphorylation of regulatory factors downstream, regulating the expression of genes involved in the formation of biofilms (Sturme et al., 2002).

Biofilms are significant in various fields, such as medicine, bioremediation, and environmental applications. However, biofilms in food processing environments can cause economic losses by food spoilage and disease outbreaks, raising food safety concerns (Kumar & Anand, 1998; Srey et al., 2013; Van Houdt & Michiels, 2010). Consequently, the food industry dedicates research efforts to comprehend biofilm formation, interventions, and mitigation strategies.

1.6 Exploring Biofilm Formation on Food Processing Surfaces

Biofilm formation is a multi-stage process that results in the development of structured microbial communities protected by an outer layer. Biofilms in food-processing environments persist because of their response to different environmental factors, both living and non-living (Bridier et al., 2015; Jefferson, 2004; Shi & Zhu, 2009). Crucial biotic factors for biofilm formation involve microbial species, cell-to-cell communication, metabolic activity, growth phase, interactions with other microorganisms, and genetic regulation (Garrett et al., 2008; Oliveira et al., 2015; Scallan et al., 2011). In food-processing environments, biofilm formation is influenced by abiotic factors like temperature, surface characteristics, nutrient availability,

pH, water activity, and exposure to disinfectants and antimicrobial agents (Donlan, 2002; Kumar & Anand, 1998; Shi & Zhu, 2009).

Biofilm formation starts when microorganisms attach to surfaces, which can happen actively or passively based on different factors like interfaces, cell surfaces, and motility (Donlan, 2002; Kumar & Anand, 1998). The attachment comprises reversible and irreversible stages, with the initial attachment being reversible because microorganisms have not yet committed to the differentiation process that leads to biofilm formation (Jefferson, 2004; Kumar & Anand, 1998). Attachment is influenced by surface properties like texture, hydrophobicity, pH, nutrients, water activity, and exposure to stress agents (Van Houdt & Michiels, 2010). Unlike smooth surfaces, rough surfaces encourage biofilm attachment by reducing shear forces (Donlan, 2002). Microbial cell attachment is affected by the hydrophobic nature of their surfaces. Microbes often adhere to surfaces that have similar characteristics. If their surfaces are hydrophilic, they might prefer hydrophilic surfaces as well, and the same goes for hydrophobic surfaces (Jefferson, 2004; Madsen et al., 2016; Pagán & García-Gonzalo, 2015).

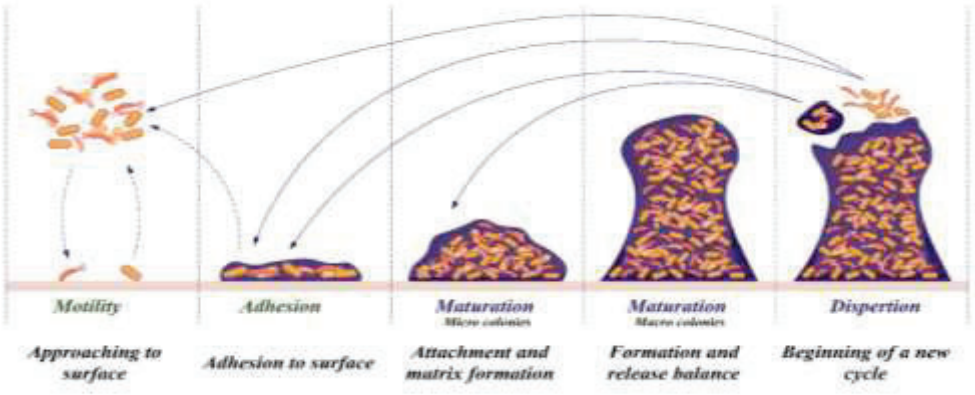


Figure 1. Biofilm formation consists of five distinct stages: Adapted from (Yin et al., 2019)

Table 2: Biofilm formation and development-related genes. (http://www.uniprot.org/) (http://biocyc.org/ECOLI) (G. Sharma et al., 2016)

Gene	Encoded Protein	Function	Location	Mass (in Daltons)
csgD	Curli fimbriae regulator	Controls the production of curli fimbriae and aids in stress management in forming biofilms	Inner cell membrane, peripheral protein	24,935
hha	Haemolysin expression modulator	Suppresses the activity of fimbriae genes, reducing biofilm formation	Cytoplasm	8,628
bcsA operon	Cellulose synthesis enzyme	Catalyzes cellulose formation, offering mechanical and chemical protection to the cell	Inner cell membrane, multi-pass protein	99,785
pgaC	PGA polymer synthesizer	Produces PGA polymer, facilitating biofilm adhesion	Inner cell membrane, multi-pass protein	50,766
fimB	Type 1 fimbriae regulator	Controls the production of type 1 fimbriae, aiding in bacterial attachment	Cytoplasm	22,993
agn43	Antigen 43	Cell-to-cell aggregation and biofilm formation in E. coli. Facilitates autoaggregation and biofilm formation through promoting interactions between cells.	Outer member protein	43,000
cah	Carbonic anhydrase	Catalyze the reversible hydration of carbon dioxide to bicarbonate ions, which plays a critical role in pH regulation	Either cytoplasmic or periplasmic	varies
ehaA, ehaB, ehaD, ehaG	EhaA, EhaB, EhaD, EhaG	Involved in adhesion and biofilm formation in EHEC	Outer membrane or periplasmic space	varies
saa	Saa protein	Initial attachment phase of biofilm formation in *Staphylococcus aureus* and promotes surface adhesion and biofilm formation.	Cell surface or extracellular matrix	varies
sab	Sab protein	Biofilm formation and virulence	Cell surface	varies

		in *Staphylococcus aureus* and contributes to surface adhesion and aggregation.	or extracellular matrix	
esp		Involved in adherence and biofilm formation in *Enterococcus faecalis* and promotes surface attachment and aggregation.	Extracellular matrix	varies

Various factors regulate biofilm maturation, including nutrient availability and QS, a cell-to-cell communication strategy (Kuchma et al., 2005; Stojicic et al., 2013). Mature biofilms have complex architectures characterized by either multi layers of microcolonies or monolayers of cells, depending on the activity of QS (Burmølle et al., 2014). Inhibiting the colonization of planktonic cells is a crucial function of rhamnolipid surfactants produced within the biofilm, which helps maintain the biofilm's structure (Davey et al., 2003; Rickard et al., 2003). There are many genes related to biofilm formation and development and some of these are outlined in Table 2.

1.6.1. Autotransporters and Their Role in Biofilm Development

Autotransporter adhesins in the type V secretion system are crucial for auto aggregation and biofilm formation during biofilm maturation. Among the autotransporter genes found in STEC strains, *agn43*, *cah*, *ehaA*, *ehaB*, *ehaD*, *ehaG*, *saa*, and *sab* are located on the chromosome, while *esp* is on plasmids (Herold et al., 2009; Puttamreddy et al., 2010; Torres et al., 2002; Wells et al., 2008). The formation of biofilms has consistently been associated with these genes and their protein products (Herold et al., 2009; Puttamreddy et al., 2010; Torres et al., 2002; Wells et al., 2008). Variability in the presence of autotransporter genes (*agn43*, *cah*, and *ehaA*) was found across different serotypes in a study involving 51 STEC strains, with non-O157 strains having a higher occurrence of *agn43*, whereas O157 strains had a greater

abundance of *cah*. (Biscola et al., 2011).

In addition, the pO157 plasmid encodes the enterohemolysin translocator *ehxD*, which has been found to play a crucial role in biofilm formation, highlighting the importance of pO157 in biofilm development (Puttamreddy et al., 2010). *EspP and ehxD* are encoded by similar large plasmids in non-O157 EHEC strains (Brunder et al., 1999; Caprioli et al., 2005; Verstraete al., 2013).

1.6.2. EPS as a Critical Factor in Biofilm Maturation

The matrix of *E. coli* biofilms comprises three distinct EPSs: poly-N-acetyl glucosamine (PGA), colanic acid, and cellulose (Vogeleer et al., 2014). Although the genomes of STEC strains EDL933 and Sakai contain the genes for synthesizing these polysaccharides, their direct impact on biofilm formation remains to be fully known (Hayashi, 2001; Perna et al., 2001). Recent studies have provided information about the governing mechanisms behind biofilm formation including the dual function of the McaS small RNA in activating PGA synthesis and controlling biofilm formation (Bond et al., 2021; DePas et al., 2014; Hufnagel et al., 2016), As well, the role of type I fimbriae, adhesin AG43, PGA, and curli fibers in is better understood in various stages of biofilm formation by pathogenic *E. coli*. (Winans et al., 2022). Mutant O157:H7 that do not possess PGA, cellulose, or colanic acid genes lack the ability to adhere to alfalfa sprouts, implying a role of these factors in biofilm adherence (Matthysse et al., 2008). In addition, cellulose production has been linked to the formation of biofilms in O157 strains. However, the extent of this correlation varies depending on the bacterial strain and environmental factors (Biscola et al., 2011; Lee et al., 2011). Although colanic acid production by *E. coli* O157:H7 confers protection against osmotic and oxidative stress. (Beloin et al., 2008; Yeh & Chen, 2004). In a recent study, found that the protection

offered by colanic acid production in *E. coli* doesn't always increase when treated by chlorine water. Whether this survival depends on the environment where the attachment occurs, type of vegetable and chlorine concentrations (Lee et al., 2016).

1.6.3. Additional Key Factors in Biofilm Development

Apart from autotransporters and EPS, lipopolysaccharides (LPS) and capsules have been associated with *E. coli* biofilm formation (Vogeleer et al., 2014). Mutations altering LPS production lower the ability of *E. coli* to bind to surfaces and develop biofilms (Beloin et al., 2008; Genevaux et al., 1999). Biofilm formation is indirectly influenced by capsules, which are recognized for their ability to disguise bacterial surface adhesions (Schembri et al., 2004). Although the impact of certain capsule types on biofilm formation by STEC remains unexplored, capsules produced by certain EHEC strains have been identified as belonging to the *E. coli* group 4 capsule (Whitfield, 2006). Further investigation is necessary to understand the role of this capsule type in biofilm formation and its expression under laboratory conditions.

Furthermore, curli fimbriae, which gather on cell surfaces, have been connected to the ability of STEC to adhere to human cells and create biofilms on inanimate surfaces (Cookson et al., 2002; Olsén et al., 1989; Uhlich et al., 2006). Although not tied to serotype, curli expression may not be necessary for biofilm formation in all cases (Wang et al., 2012). Additionally, curli could interact with cellulose, resulting in the formation of networks that create a hydrophobic outer layer (Zogaj et al., 2001). Curli fibers are thought to assist in both initial and subsequent interactions between cells and surfaces (Cookson et al., 2002; Ryu et al., 2004; Uhlich et al., 2006).

The bacterial cell population density changes and gene expression fluctuates throughout

the different stages of biofilm formation. Bacteria utilize QS systems to coordinate gene expression (Walters & Sperandio, 2006) and some of the genes contributing to this mechanism are outlined in Table 3. Autoinducers (AIs) are signal molecules that QS systems use for secretion and recognition. AI-1, AI-2, and AI-3 are the three types of AIs identified. Both AI-2 and AI-3 are produced, secreted, and recognized by *E. coli* strains, including STEC (Walters & Sperandio, 2006). *E. coli* strains do not produce AI-1. This enables *E. coli*, including STEC strains, to recognize AHL, the signal molecule for AI-1, secreted by other bacterial species. Sharma et al. (2010) revealed that *SdiA* is a curli and flagellar gene expression repressor. The enzyme *LuxS*, present in STEC strains, is crucial in converting ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione. This compound is the precursor for AI-2 (Schauder et al., 2001). Adding AI-2-like molecules to an O157:H7 *luxS* deletion strain increased biofilm formation (Lu et al., 2005). The QseBC two component system recognizes AI-3 and host produced epinephrine/norepinephrine (Walters & Sperandio, 2006). Epinephrine and norepinephrine enhance STEC motility and biofilm formation, but indole reduces these effects (Bansal et al., 2007). Additionally, the *qseC* deletion strain showed a 50% decrease in motility and biofilm formation compared to the wild type of strain (Yang et al., 2014).

Table 3: Genes involved in Quorum Sensing in *E. coli.* (http://www.uniprot.org/) (http://biocyc.org/ECOLI) (Brito et al., 2013; González Barrios et al., 2006; Sharma et al., 2016)

Gene Name	Protein Produced	Function	Location	Mass (in Daltons)
luxS	S-Ribosylhomocysteine Lyase	Makes AI-2, which helps in forming biofilms and controlling their structure	Inside the Cell (cytoplasm)	19,416
mqsR	mRNA Interferase MqsR	Controls how cells move together and	Inside the Cell (cytoplasm)	11,232

		positively influences qseBC activity		
qseB	Transcriptional Regulator QseB	Affects the creation of flagella (the tiny tails that help bacteria move), activates certain genes, and controls cell movement	Inside the Cell (cytoplasm)	24,678
qseC	Sensor Protein QseC	Acts like a sensor, detecting certain chemicals outside the cell, and might help in turning on certain genes	Inside the Cell (cell inner membrane)	50,282
pfs	5'-Methylthioadenosine/S-Adenosylhomocysteine Nucleosidase	Helps in making AI-2 and regulates the assembly of flagella (tiny tails)	Inside the Cell (cytoplasm)	24,354
flhD	Flagellar Transcriptional Regulator FlhD	Controls the production of flagella, which helps bacteria move around	Inside the Cell (cytoplasm)	13,316
fliA	RNA Polymerase Sigma Factor FliA	Provides the energy needed to spin flagella (the tiny tails that help bacteria move)	Inside the Cell (cytoplasm)	27,521
motA	Motility Protein A	Helps process AI-2 and release certain repressors, allowing cells to move more freely	Inside the Cell (cell inner membrane)	32,011
lsrK	Autoinducer 2 Kinase LsrK	Helps control the movement of certain molecules in and out of the cell, based on the presence of AI-2	Inside the Cell (cytoplasm)	57,545
lsrR	Transcriptional Regulator LsrR	Affects how certain genes are turned on or off based on the levels of AI-2 present in the cell	Inside the Cell (cytoplasm)	33,797
csrA	Carbon Storage Regulator	Affects how cells store energy and influence the creation	Inside the Cell (cytosol)	6,856

		of flagella		

(Note: Protein locations indicated have been verified using the PSORT database, a predictive tool for determining protein location in bacterial cells.)

Dispersion is the last step of biofilm formation, enabling cells to return to a planktonic state (Chua et al., 2014). Bacterial cells respond to stress and nutrient depletion by adjusting their intracellular signaling molecule, c-di-GMP, which triggers dispersion (Donlan, 2002; Jefferson, 2004). Bacteria can colonize new niches and find nutrient rich environments through detachment (Madsen et al., 2016). During dispersion, the transition period is crucial because dispersed cells may become more virulent before finding new environments (Chua et al., 2014).

Biofilms typically develop in shared spaces like floors, drains, water pipes, and hard-to-clean surfaces in food-processing settings (Dass & Anandappa, 2017; Wang, 2019) increasing the chances of foodborne illness (Bogino et al., 2013; Fox et al., 2014). Furthermore, STEC can form robust biofilms on different surfaces in meat processing facilities, such as conveyor belts and equipment (Rivera-Betancourt et al., 2004; Stopforth et al., 2003). Biofilms and their resistance to sanitizers contribute to the persistence of these pathogens in the meat processing environment (Wang et al., 2014).

The switch from reversible to irreversible attachment is a significant stage in the development of biofilms, marked by the reinforcement of bacterial bonds and the covering of cells in an EPS layer (Madsen et al., 2016; Stoodley et al., 2002). The presence of diverse biopolymers in EPS boosts the resilience of biofilms against environmental stresses and antimicrobial agents (Donlan, 2002; Joshi et al., 2021). Because of their resilience, biofilms often require mechanical force, elevated temperatures, detergents, and sanitizers for removal (Turnbull et al., 2016; Wang et al., 2016; Wu et al., 2015).

1.7 Architecture of Biofilm

Both single-species and multi-species microbial communities can form biofilms. Different microorganisms exhibit varying abilities to form biofilms, which multiple factors can influence. For instance, a bacteria may be highly proficient at producing biofilms in one setting but less in another (Davey et al., 2003). Biofilm structure is influenced by various factors such as surface properties, nutrient availability, microbial community composition, and hydrodynamics (Tan et al., 2014).

Regardless of being composed of one or multiple species, biofilms generally display similar structural features (Donlan, 2002). Coaggregation is the initial step in forming a biofilm, where genetically diverse bacteria adhere to one another through adhesins and receptors on their cell surfaces (Afonso et al., 2021; Ren et al., 2015; Wu et al., 2015). There are two ways in which coaggregation can take place: planktonic cells can recognize and adhere to genetically distinct cells in the developing biofilm, or secondary colonizers can first aggregate and then adhere to the biofilm (Ledder et al., 2008; Rickard et al., 2003). Co-adhesion leads to the integration of adhered cells into the biofilm community (Rickard et al., 2003; Taga & Bassler, 2003).

Biofilms have fluid channels known as interstitial voids enclosed in an EPS layer (Jefferson, 2004). These empty spaces are vital elements of the biofilm structure and aid in transferring nutrients, oxygen, waste, and antibiotics (Davey et al., 2003; Stoodley et al., 2002). Figure 2 demonstrates how interstitial voids in a mixed species drain biofilm enable fluid flow (Chitlapilly Dass & Wang, 2022).

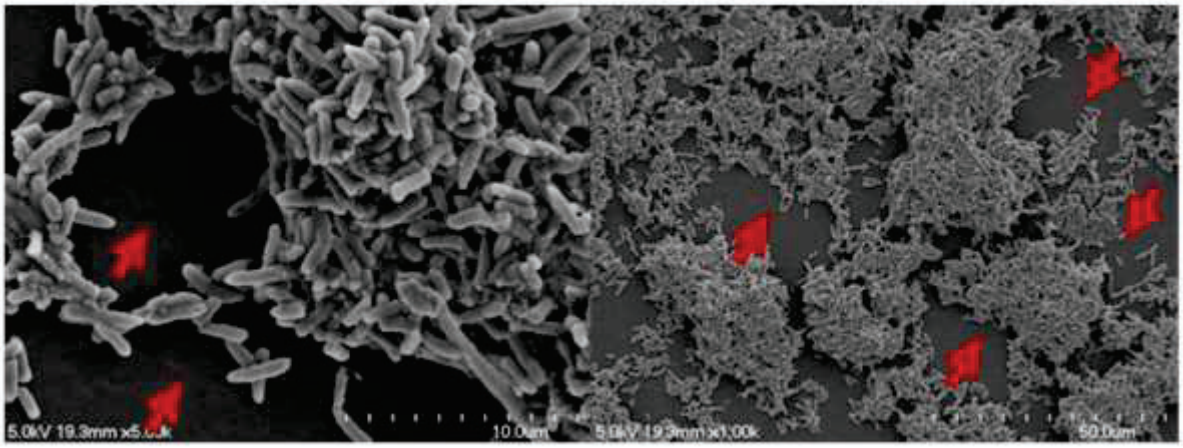


Figure 2: SEM image shows interstitial voids in a mixed species drain biofilm on a stainless-steel chip after 5 days at 7°C. The interstitial voids are highlighted by the red arrows. Image source (Chitlapilly Dass & Wang, 2022).

Oxygen is distributed unevenly within biofilms, with lower oxygen levels detected in deeper layers (Kühl et al., 2007; Taga & Bassler, 2003). Respiring bacteria consuming oxygen can form anoxic areas within biofilms, extending beyond bacterial cells. As microcolonies develop, the oxygen concentration rapidly decreases from the surface to the interior, causing the induction of anaerobic respiration genes in the interior regions (Karampatzakis et al., 2017; Tan et al., 2014). In addition, the depth of biofilms leads to a decrease in nutrient concentration because of consumption and the increased distance from the nutrient source (Fuqua et al., 2001; Stewart & Franklin, 2008). Moreover, the properties of nutrient buildup in biofilms of natural microbial groups vary based on nearby nutrient supplies (Sztajer et al., 2014).

1.8 STEC Super Shedders

The phenomenon of “super shedding (SS)” animals is a significant source of STEC contamination in cattle farming (Castro et al., 2022). These animals can expel highly concentrated levels of STEC, often exceeding 10,000 (CFU/g) of stool, earning them the

designation of SS. Studies show that SS of *E. coli* O157:H7 in feces by these animals is sporadic, typically lasting for less than a month and ranging from 10 to 10^7 CFU/g of feces (Besser et al., 1997; Stephens et al., 2008).

Researchers believe the mechanism underlying animal SS is associated with STEC biofilms forming within the intestinal epithelium (Munns et al., 2015). According to longitudinal studies, SS among cattle herds varies in duration and prevalence, with reported rates ranging from 0.48% to 71% of animals (Munns et al., 2015). Differences in animals, diet, study duration and timing contribute to this variation (Chase-Topping et al., 2008). Additionally, SS animals have a more diverse intestinal microbiome than non-SS animals (Xu et al., 2014). Non-SS animals exhibit more effective immune protection in rectal tissues than SS animals, indicating potential differences in host defense mechanisms (Wang et al., 2016).

Despite attempts to comprehend and manage SS, obstacles remain (Castro et al., 2022). Most studies concentrate on O157:H7, but there is some research on non-O157, including SS events (Murphy et al., 2016). It is still uncertain whether there is a link between animals that shed excessively and contamination of carcasses. While some research indicates that SS play a significant role in contamination, other studies show minimal transmission between them and their pen mates (Matthews et al., 2006). Efforts to reduce STEC prevalence by identifying SS animals have had limited success, as removing them from herds has shown minimal impact on contamination dynamics (Munns et al., 2015).

Investigating the potential link between SS events and biofilm formation in the RAJ has become an emerging area of interest. Several investigations have shown that *E. coli* strains can form biofilms on various surfaces, with multi-species biofilms being frequent (Wang et al., 2016). The higher microbial diversity in SS fecal samples may be due to multi-species biofilms (Xu et al., 2014; Dixon et al., 2020; Murphy et al., 2016).

A comprehensive approach is needed to determine the factors impacting SS dynamics, which include pathogen, host, and environmental factors. Stress induced immune suppression may play a role in the increased shedding dynamics associated with stressors such as sunlight exposure and heat stress (Venegas-Vargas et al., 2016). Additionally, shedding dynamics may be affected by dietary factors and fecal consistency, with increased shedding linked to grain-based diets and lower fecal water content (Beauvais et al., 2018). The investigation should also consider the influence of environmental factors, such as seasonality and climatic conditions, on shedding dynamics (McCabe et al., 2019).

1.9 STEC High Event Periods

Beef processors define "high-event periods" (HEPs) as a notable increase in *E. coli* O157:H7 contamination. These levels are considerably above the typical 5% baseline for bacterial detection (FSIS 2014). HEPs pose health risks and economic burdens for the industry (Wells, 2021). According to researchers, HEPs can be caused by various factors, including weather changes, seasonal variations, animal stress levels, SS cattle, and biofilms (Arthur et al., 2014). Among these factors, biofilms present a complicated challenge. Low bacterial diversity in HEP incidents may be associated with biofilms, indicating contamination by a single, persistent strain (Arthur et al., 2014). The occurrence of HEPs and contamination spikes may be caused by the release of bacteria from biofilms during their lifecycle (Lim et al., 2017; Srey et al., 2013).

Research has highlighted the connection between biofilms and HEPs, showing that strains of *E. coli* O157:H7 associated with HEPs have a higher capacity for forming biofilms than other strains (Marouani-Gadri et al., 2009). The biofilms and their sanitizer resistance significantly contribute to HEP occurrence (Wang et al., 2016). Biofilm bacteria can detach

and infect meat products, resulting in outbreaks that lack identifiable patterns or links to process control failures (Wang et al., 2014).

1.10 Potential Impacts of SS or HEP on Food Chain

Super shedder events and HEPs have a complex impact on the food chain, raising concerns about environmental contamination and food safety (Castro et al., 2022). Research has indicated that SS events can contaminate animal hides, and feedlots with high numbers of SS often have high levels of hide contamination (Arthur et al., 2009). Because of contaminated hides, there is a direct risk of carcass contamination during slaughter, which could cause foodborne illnesses. Although efforts have been made to minimize fecal contamination on carcasses, studies indicate that contamination between hides and carcasses cannot be eliminated, underscoring the necessity of effective microbiological control measures (Stephens et al., 2008).

The difficulty of ensuring safe products is emphasized by exceptionally high concentrations of O157:H7 in naturally colonized SS steers and their feedlot environment (Stephens et al., 2008). Biofilm-producing strains in meat processing facilities persist and aggravate the problem, as studies have found that these strains can contaminate surfaces repeatedly for as long as 30 days (Wells, 2021).

Moreover, there is a notable difference in the ability to form mature biofilms between isolates obtained during HEPs and unrelated strains. There seems to be a positive correlation between HEPs and biofilm formation on surfaces used for meat production (Wang et al., 2014). The unclear relationship between SS events and HEPs raises questions about the origin of these strains, as observed by the low diversity of genotypes (Arthur et al., 2014). SS events could influence HEPs, but it is also likely that a single strain capable of forming biofilms can

become established on meat-processing equipment, leading to HEPs (Castro et al., 2022), independently of SS. However, not all outbreaks of food-borne disease have been linked to HEPs, as other undetermined factors could be contributing to these incidents.

1.11 Control Measures for Preventing *E. coli* Biofilm Formation

E. coli poses a significant threat in food processing due to its ability to form resilient biofilms on various surfaces, such as food, pipes, and equipment (Duc et al., 2018). These biofilms make *E. coli,* including STEC, more resistant to environmental conditions and reduce disinfectant effectiveness, raising concerns about food safety and consumer health (Zhou et al., 2022). Therefore, it is crucial to implement effective strategies to minimize or control *E. coli* biofilm formation during processing to mitigate the risk of microbial contamination.

1.11.1. Thermal

Thermal processing is crucial for reducing the risk of pathogenic contamination in food, especially with pathogens like *E. coli* O157:H7 (Erickson & Doyle, 2007). Studies on this pathogen's heat sensitivity have gained valuable insights, with researchers extensively exploring factors such as D-values and Z-values (O'Bryan et al., 2006). *E. coli* O157:H7 is not considered to be heat resistant (Kaur et al., 1998), being more heat sensitive than indigenous beef bacteria (Juneja et al., 2003) and more sensitive than Salmonella and Listeria in pork (Murphy et al., 2004) and chicken-fried beef patties (Osaili et al., 2006).

The composition of food impacts the pathogen's ability to tolerate heat. Furthermore, the fat content in beef has been shown to affect *E. coli* O157:H7's heat resistance, with higher fat content resulting in improved thermal resistance due to reduced thermal conductivity and water activity (Smith et al., 2001).

Different cooking methods have been studied to see how well they kill pathogens, but none offer perfect control. Microwave heating, while efficient in terms of speed, suffers from non-uniform heating, leading to the potential survival of *E. coli* O157:H7 in seemingly well-cooked products (Apostolou et al., 2005). Thorough cooking practices are essential, grilling may not always guarantee pathogen inactivation, particularly if single-sided (Apostolou et al., 2005).

Erickson & Doyle (2007) emphasized validating thermal processes and creating mild heat treatments to eliminate pathogens. Various chemical additives, like ozone or acidulants, have demonstrated potential for improving thermal inactivation, although their success can vary based on factors such as food type and additive concentration (Novak & Yuan, 2003). Despite this, the concerns regarding changes in carcass coloration or increased pathogen virulence due to thermal treatments are primarily theoretical, with limited practical evidence to back up these assertions (Huang, 2004). High-temperature methods, including superheated steam demonstrate superior efficacy in inhibiting *E. coli* biofilm formation on surfaces like stainless steel (Ban et al., 2014).

1.11.2. Chemicals

Common disinfectants used in food processing, such as chlorine-based agents, quaternary ammonium compounds, and lactic acid, can partially inhibit *E. coli* biofilm formation but often fall short of complete eradication (Wang et al., 2016). Recent studies have explored alternative chemicals like chlorine dioxide and neutral oxygen potential water, showing promising inhibitory effects on *E. coli* biofilms (Pinngoen et al., 2016). Interestingly, the effectiveness of disinfectants varies depending on the surface material, with stainless steel exhibiting higher anti-biofilm activity than other surfaces like plastic or wood (Bang et al.,

2014). However, traditional chemical disinfectants face challenges in adequately penetrating biofilms, and their use may negatively impact food properties and consumer health (Zhou et al., 2022). Photodynamic sterilization, utilizing riboflavin-mediated technology, emerges as a promising approach. It effectively inhibits *E. coli* biofilm formation by inducing bacterial oxidative stress (Banerjee et al., 2020).

1.11.3. Freezing

In contrast to other methods, freezing does not significantly reduce pathogen cell numbers. For instance, Conner and Hall (1994) found that 50% of *E. coli* O157:H7 cells survived 18 months of storage at -20°C in ground chicken breast meat. Freezing may not significantly decrease pathogen numbers, but it can cause damage and reduce viability in certain situations. Various factors can intensify these effects, such as multiple freeze-thaw cycles (Yamamoto & Harris, 2001) or frozen storage with chemical additives (Ingham et al., 2006; Uljas & Ingham, 1999). Moreover, the pathogen's growth conditions can influence freezing's impact on *E. coli* survival. Cold stress, such as exposure to 4°C for 4 weeks or starvation, where cells are suspended in water at 37°C for 6 hours, has been shown to increase the survival of *E. coli* O157:H7 during freeze-thaw cycles (Elhanafi et al., 2004; Gawande & Griffiths, 2005). Furthermore, innovative technologies such as low-temperature plasma sterilization exhibit promising anti-biofilm activities while preserving food quality (Kovalova et al., 2016). Also, maintaining lower temperatures in food processing environments can reduce *E. coli* transfer and minimize microbial cross-contamination (Adator et al., 2018). These findings emphasize the complexity of freezing as a pathogen control method and the need to consider multiple factors in food safety protocols.

1.11.4. Physical

In addition to chemical approaches, physical methods like vibration techniques are crucial in controlling *E. coli* biofilms. Research indicates that the application of nano-vibrations to material surfaces can effectively impede the formation of *E. coli* biofilms (Lin et al., 2021). These results may enable development of approaches for cleaning mechanical surfaces in food or medical settings.

Combining chemical and physical methods synergistically enhances microbial control in food processing. For instance, combining lactic acid with water vapor results in potent antimicrobial effects, reducing *E. coli* abundance on various surfaces (Ban et al., 2014). Similarly, cold nitrogen plasma combined with clove oil shows significant synergistic inhibition of *E. coli* biofilms (Cui et al., 2016).

1.11.5. High-Pressure

High-pressure methods, like high hydrostatic pressure (HHP), are a promising technology for killing pathogens in food using pressures ranging from 100 to 800 MPa to liquid and solid foods (Erickson & Doyle, 2007). The mechanism of inactivation by HHP involves multiple targets, including the cytoplasmic membrane, leading to solute loss, protein coagulation, enzyme inactivation, and changes in ribosome conformation (Mañas & Pagán, 2005). Inactivation of *E. coli* O157:H7 in whole milk exhibited a logarithmic relationship with treatment pressures within the range of 450 to 690 MPa, although strain-dependent variations in resistance were observed (Benito et al., 1999; Malone et al., 2006). Rapid decompression steps or dynamic high-pressure techniques with multiple exposures to reduced pressures can be utilized to improve the effectiveness of high-pressure treatments. For example, rapid decompression following a 250-MPa treatment of orange juice resulted in a more significant

reduction of viable *E. coli* O157:H7 compared to slow decompression methods (Noma et al., 2004). It is crucial to acknowledge that HHP treatments may result in sublethal injury, as cells undergo a recovery process to repair cell damage and to avoid overestimating microbiological safety (Bozoglu et al., 2004).

1.11.6. Pulse Electric Fields

Cell death in pulse electric field (PEF) interventions is mainly caused by membrane structure or function changes resulting from high-voltage pulses (5 to 80 kV/cm) to foods (Mañas & Pagán, 2005). For instance, exposing dialyzed egg white, egg yolk, and whole egg products at 0°C to 500 pulses with an electric field strength of 15 kV/cm reduced *E. coli* O157:H7 by 1, 3, and 3.5 log CFU/g, respectively (Amiali et al., 2004). Moreover, studies have shown that increasing treatment temperature enhances the *E. coli* O157:H7 inactivation rate in both liquid egg white (Amiali et al., 2006) and liquid whole egg (Bazhal et al., 2006). Exposing sublethal-injured cells to an acidic environment after PEF treatment can also enhance inactivation (García, Gómez, et al., 2005). For example, treating apple juice with PEF at 25 kV/cm for 400 s and subsequently storing it under refrigeration for 48 h resulted in a 5-log CFU/g reduction of *E. coli* O157:H7 compared to a 1-log CFU/g reduction immediately after treatment (García, Hassani, et al., 2005).

Application of PEF to solid foods is still in its infancy phase compared to its application to liquid foods (Zhang et al., 2023). For example, PEF treatment of solid foods for preservation may be unrealistic, as most species of microorganisms are not affected by low-intensity PEF treatment (Peng et al., 2020). PEF treatment in solid foods such as meat faces difficulties due to the electrical resistance of solid foods, leading to inconsistent treatment results (Aşık-Canbaz et al., 2022). The resistance of food materials varies depending on their

water and salt contents. Oils and fats have resistances exceeding 100 Ω (Gudmundsson & Hafsteinsson, 2005). The low conductivity and high protein fat content of meat products restrict the usefulness of PEF in this context (Alahakoon et al., 2016).

1.11.7. Sound Waves

Sound waves are applied to foods in a liquid to generate intense pressure and heat, destroying microbial cells. While ultrasonography has been successfully used to remove bacteria from poultry carcasses in chlorinated water (Lillard, 1994), applying it to larger carcasses like beef or swine is not feasible due to the requirement of liquid immersion. However, in liquid foods such as apple juices, continuous flow ultrasound treatment, and mild heat effectively reduced *E. coli* O157:H7 cell numbers by 6 log CFU/ml (D'amico et al., 2006).

1.11.8. Irradiation

Low and medium doses of irradiation (ranging from 1 to 2 kGy) applied to *E. coli* O157:H7 contaminated beef steaks and ground beef successfully reduced the pathogen loads by 4 to 5 log CFU/g without adverse effects on flavor and color (Arthur et al., 2005; Fu et al., 1995). Similarly, medium dose irradiation treatment (2.47 kGy) of apple cider reduced acid resistant *E. coli* O157:H7 by 5 log CFU/ml (Bazhal et al., 2006). The effectiveness of irradiation treatments depends on the type of food. Inactivation of O157:H7 was more effective in chicken than beef or trout and significantly greater in broccoli than alfalfa seeds (Rajkowski et al., 2003; Thayer et al., 2003). However, doses exceeding 2 kGy can negatively impact sprouting and subsequent yield, necessitating treatment combinations like irradiation (2.0 kGy) with dry heat at 50°C for 60 min to ensure complete inactivation of *E. coli* O157:H7

on alfalfa seeds without compromising germination (Bari et al., 2003).

Low dose ionizing radiation interventions have successfully eliminated harmful bacteria in food by creating thymine dimers and toxic free radicals. While commercial use of food irradiation is limited, research aims to reduce adverse quality effects by evaluating low and medium dose treatments (Erickson & Doyle, 2007).

1.11.9. UV Light

Applying germicidal ultraviolet (UV) light (wavelength ranges of 100 to 280 nm) on surfaces and liquid foods prevents bacterial multiplication by altering pyrimidine bases in bacterial DNA (Erickson & Doyle, 2007). UV light treatments have effectively reduced *E. coli* O157:H7 on various food surfaces and liquid media. For example, UV light (253.7 nm) applied at a dosage of 24 mJ/cm^2 reduced *E. coli* O157:H7 on apples and leaf lettuce by approximately 3.3 and 2.79 log CFU/g, respectively (Yaun et al., 2004). UV doses of 14 mJ/cm^2 in apple cider achieved a 5-log CFU reduction of *E. coli* O157:H7/ml, varying effectiveness based on the strain and apple cultivar used (Basaran et al., 2004). The effectiveness of UV light in killing microorganisms can be enhanced by pulsing the light source (Dunn et al., 1995).

Treatment with UV light has been studied by researchers in recent years for its ability to deactivate microorganisms in beef (Wang et al., 2023). Söbeli et al., (2021) explored the impact of pulsed light (PL) at different dosages in beef tenderloin steak. Under the PL treatment at 4.2 J/cm^2, there was a substantial reduction of 3.49 ± 0.67 log CFU/g in aerobic mesophilic bacterial count. In contrast, Dunn, 1997 used PL at 5 J/cm^2 to sterilize raw beef surfaces, resulting in less effective microbial inactivation than Söbeli et al. (2021). Difference in sample surface characteristics could explain this discrepancy, as smoother surfaces allow

for more contact between microorganisms and UV light, increasing their exposure to UV radiation (Dunn, 1997). Likewise, Hierro et al. (2012) found PL reduced 0.9, 1.2, and 1.0 log CFU/cm^2 were observed for *L. monocytogenes*, *E. coli* and *S. typhimurium* respectively Bryant et al. (2021) explored the effects of PL treatment on *Escherichia coli* K12 inactivation on beef surfaces and found increased microbial inactivation rates were observed with longer treatment times and shorter distances between the UV light and the sample.

Generally, the more area exposed to UV, the greater the reduction of microbes and the effectiveness of UV treatment on beef is mainly determined by the exposed surface area. Nevertheless, extended UV exposure can negatively impact the sensory attributes of beef (Wang et al., 2023). Therefore, researchers propose the combination of UV treatment and LED technology to improve beef quality after UV treatment (McSharry et al., 2022; Shebs et al., 2022). Although UV treatment may result in slight alterations in meat color and sensory characteristics, careful adjustment of treatment parameters can minimize these impacts, guaranteeing customer approval (Hierro et al., 2012).

1.11.10. Biological

With conventional disinfectants showing decreased efficacy against biofilms (Zhou et al., 2022), biological extracts offer a promising alternative due to their safety and environmental friendliness. Extracts from natural sources like animal shells, bacteriophages, and plant compounds exhibit notable inhibitory effects on *E. coli* biofilms (Zhou et al., 2022). For instance, scallop shell powder, rich in calcium oxide, demonstrates remarkable anti-biofilm activity, inhibiting *E. coli* biofilm formation on surfaces like stainless steel (Bodur & Cagri-Mehmetoglu, 2012). Bacteriophages, natural predators of bacteria, show potential in biofilm prevention and elimination, with phage AZO145A significantly inhibiting *E. coli*

biofilm formation (Wang et al., 2020). Plant extracts, mainly phenols and essential oils, exhibit inhibitory solid effects on *E. coli* biofilm by disrupting bacterial metabolic activities and inhibiting biofilm formation (Baptista et al., 2019; Cui et al., 2020; da Silva et al., 2019; Lou et al., 2013; Mohammadi et al., 2019).

1.12 Desiccation as an Antimicrobial Hurdle

Alongside other preservation techniques, salt is crucial in the food industry for its versatile role as a preservative and antibacterial agent. Its ability to prevent bacteria from growing is useful in ready to eat (RTE) meats, seafood, fermented foods like salami, cheese, baked goods, and fruits and vegetables (Burgess et al., 2016). Additionally, salt is highly valued for its capacity to improve the taste, consistency, and preservation of meat products (Ruusunen & Puolanne, 2005). Salt's action involves damaging bacterial cells by disrupting the balance between their internal and external environments (Csonka, 1989).

Cell desiccation, characterized by bulk water removal, presents unique challenges and opportunities in food preservation, surface disinfection, and the production of dried cultures for industries like dairy, beer, and wine (Nocker et al., 2012). The desiccation process causes various cellular changes, such as shrinkage of the capsular layer, salt accumulation within the cell, and concentration of macromolecules due to decreased cell volume (Potts, 1994). Furthermore, desiccation modifies biophysical features, lowers membrane fluidity, and causes harm to proteins and DNA frequently by attacking free radicals (Shirkey et al., 2000). The higher resistance to desiccation in Gram-positive bacteria is attributed to differences in cell structure compared to Gram-negative bacteria (Miyamoto-Shinohara et al., 2008).

Koster (1991) discovered that certain sugars, like disaccharides and extracellular polysaccharides, can guard against desiccation by creating highly viscous supersaturated solid

solutions. By maintaining hydrogen bonding at the sugar solution-cell interface, these solutions prevent cellular collapse and preserve membrane integrity and protein structure (Welsh, 1999). Moreover, extracellular polysaccharides play a critical role in biofilm formation and safeguarding cells from drying out by hydraulically decoupling them (Potts, 1994). The concentrations of magnesium chloride have been found to affect how susceptible bacteria are to drying, especially in Gram-negative bacteria (Nocker et al., 2012).

Despite osmotic and desiccation stress, bacterial survival and multiplication in food and food-processing environments significantly contributes to their persistence and the risk of transmission to humans (Sleator et al., 2003). Nevertheless, exposure to osmotic stress could potentially offer protection against subsequent stresses, including low temperature or bile salts (Begley et al., 2002). Bacteria can adapt to stressful environments by accumulating compatible solutes (Jørgensen et al., 1995).

1.13 Effect of Temperature and Time on Survival of STEC

Studies have revealed that *E. coli* O157:H7 can linger in bovine feces for quite some time, ranging from 49 to 126 days at a moderate temperature of 15°C (Duffy, 2003; Fukushima et al., 1999). Although farming methods differ, the survival of STEC in feces remains primarily unchanged. Research indicates that *E. coli* O157:H7 and O26:H11 strains can endure for up to 90 days in cattle slurry or manure (Avery et al., 2005; Fremaux et al., 2007; McGee et al., 2001; Nicholson et al., 2005). Temperature determines the fate of STEC in cattle effluents. Warmer temperatures tend to hasten the decline of *E. coli* O157:H7, with viable levels diminishing within 5 days at 23°C. In contrast, STEC can persist for over 28 days at lower temperatures of 4 or -20°C (Kudva et al., 1998). The decrease is caused by less microbial activity at colder temperatures and the evaporation of effluent at hotter

temperatures. Surprisingly, despite the prevailing assumption of extended survival in colder conditions, research indicates that seasonal fluctuations do not significantly alter the decline of *E. coli* O157:H7 in cattle slurries (Hutchison et al., 2005). Nevertheless, conflicting evidence suggests that storing effluents at temperatures exceeding 20°C may augment the persistence of *E. coli* O157:H7, with survival rates doubling at 22°C compared to lower temperatures (Bach et al., 2005; Himathongkham, 1999).

The beef industry follows strict manufacturing practices, including maintaining a cold chain, to minimize spoilage and contamination (Zhang et al., 2022). The cold chain usually maintains temperatures at 10°C or below, preventing bacterial growth, including O157:H7 (Doyle & Schoeni, 1984). The impact of temperature on biofilm formation by different STEC serovars was demonstrated by an increase in biofilm mass on stainless-steel coupons from 0.14 to 0.3 relative biofilm biomass (RBB) over 72 hours at 22°C (Bumunang et al., 2020). However, the biofilm mass was lower (RBB 0.09) when the stainless-steel coupons were kept at 10°C for 168 hours. A consistent response to temperature variations was observed across various STEC serovars (Bumunang et al., 2020). Adator et al. (2018) observed that ambient temperature played a role in the differences in biofilm forming strength among various STEC serovars. Using the crystal violet optical density method, they discovered a notable growth in biofilm mass after four to six days at 25°C, while biofilm formation was minimal at 10°C. Although lower temperatures hinder the growth rate of O157:H7, Dourou et al. (2011) determined that the bacteria can still attach to surfaces and create biofilms even at temperatures as low as 4°C. Over a week at 4°C, O157:H7 demonstrated increased adherence to stainless steel coupons in a fat lean homogenate growth medium. The increase in bacterial counts was linked to factors like brownian motion aiding bacterial contact with the coupon or getting trapped in exopolymers produced by other bacteria like *Pseudomonas* spp. (Dourou et

al., 2011). Significant biofilm formation by *E. coli* O157:H7 was observed on contaminated stainless-steel coupons over time, with biofilm populations stabilizing at higher levels after 7 days at 15°C (Simpson Beauchamp et al., 2012).

Small changes in growth temperature can influence protein synthesis, affecting surface characteristics and bio-adhesive behavior. Furthermore, temperature stress may lead to adhesion and trigger a strain's adaptive response to cold temperatures (Shi & Zhu, 2009; Zeraik & Nitschke, 2012). Interestingly, high temperatures can also increase the adherent nature of biofilms to surfaces, referred to as the "baking effect" (Garrett et al., 2008).

Pompermayer and Gaylarde, (2000) found that reduced temperatures had minimal effect on biofilm formation and the total number of adherent cells was similar at 12°C and 30°C, regardless of the incubation time. Similarly, in a study conducted by Bezek et al. (2019), it was observed that there were no significant differences in biofouling values between 22°C and 37°C after 48 hours of incubation. According to other studies, it is suggested that factors associated with adhesion or other surface colonization mechanisms could have a more substantial influence than temperature variations in establishment of biofilms (Andersen et al., 2010; Barker & Bloomfield, 2000; Herald & Zottola, 1988).

Biofilms of strains O113, O145, O121, O45, and O103 have been shown to become denser on polystyrene surfaces over time (Wang et al., 2016). In contrast, another study found that the density of biofilm cells on stainless steel surfaces decreased over time (Ma et al., 2019). Previous research has shown a consistent increase in *E. coli* biofilm formation on stainless steel at 23°C (Nguyen et al., 2014) and 15°C (Dourou et al., 2011) for up to 24 hours. However, some studies have observed decreased cell density in biofilms after 48 hours (Nguyen et al., 2014).

Duffy et al. (2006) conducted a study comparing the effects of temperature on *E. coli*

O157:H7 and O26. They assessed the viability of these STEC strains in yogurt and orange juice at a temperature of 4°C and pH levels ranging from 4.1 to 4.5. According to their findings, the STEC strains stayed viable in yogurt for approximately 18 days and in orange juice for about 30 days. The ability of O157 and O26 to survive longer in refrigerated orange juice and yogurt may be due to stress proteins activated by cold and acid conditions (Duffy et al., 2006). These researchers also determined the D55-values for heat-shocked and non-heat-shocked antibiotic-sensitive and -resistant *E. coli* O157:H7 and O26 in minced beef. The findings indicated that heat-shocked O157 and O26 had higher mean D55-values than non-heat-shocked strains, implying that heat shock-induced increased thermotolerance.

Juneja et al. (1998) reported similar findings, noting increased resistance to heating at 60°C in heat shocked *E. coli* O157:H7 compared to non-heat shocked cells. The higher expression of heat shock proteins, GroEL and DnaK was associated with increased thermotolerance. It is worth noting that Duffy et al. (2006) observed that antibiotic resistant STEC O157 and O26 strains displayed greater susceptibility to temperature treatment than antibiotic-sensitive strains. They proposed that the lowered survival in heat stress conditions might be connected to mutations in *rpoS* triggered by antibiotic exposure (Juneja et al., 1998). Enache et al. (2011) investigated the heat sensitivity of different STEC serogroups. They discovered that non-O157 strains tended to be more heat-sensitive than O157:H7 strains at 56°C. Cells adapted to acid were more heat-sensitive than non-adapted cells. These results contrast to the majority of previous studies showing that acid-adapted *E. coli* O157:H7 cells exhibit increased heat tolerance, although the cause of this inconsistency is unknown.

1.14 Effect of Moisture Conditions on Survival of STEC

The humidity and temperature levels during food storage impact the formation of

biofilms by bacteria on surfaces (Liu et al., 2013; Møretrø & Langsrud, 2017). Tomičić et al. (2017) found that a relative humidity of approximately 98% resulted in the highest bacterial and yeast adhesion levels on wooden surfaces. However, when humidity was lowered to approximately 65% or 75%, adhesion was significantly reduced or completely absent, demonstrating the practical deterrent effect of specific humidity levels on microbial growth (Tomičić et al., 2020). Higher microorganism counts have been linked to increased humidity during storage (Onilude et al., 2010).

The impact of humidity levels (70%, 85%, and 90% RH) on the survival of STEC on stainless steel surfaces at 12°C for 19 days has been investigated (Møretrø et al., 2010). STEC survival significantly decreased at humidity levels of 70% and 85% compared to higher levels. At 70% humidity, the count of STEC bacteria reduced from 6 to 5.5 log CFU on stainless steel after seven days, but the reduction was less significant at 98% humidity. The remaining STEC levels at 70% humidity dropped to 1 Log CFU after 19 days. This observation emphasizes the importance of moisture in bacterial transfer (Møretrø et al., 2010).

Research shows that drying biofilms can increase bacterial transfer to food products such as cheese and bologna (Rodríguez et al., 2007). The phenomenon is caused by the weakening of capillary forces in the drying biofilm, making it easier for bacteria to be transferred to food. Therefore, the moisture content of surfaces plays a crucial role in bacterial adhesion and subsequent transfer to food products (Flemming, 1995; Tomičić et al., 2020). Due to weakened capillary forces, biofilms become more susceptible to breaking apart when they dry out (Rodríguez & Mclandsborough, 2007).

1.15 STEC as a Source of Cross-Contamination

E. coli O157:H7 can survive on surfaces such as stainless steel and plastic (Herald &

Zottola, 1988). As a result, these surfaces can contaminate food during processing (Avery et al., 2005). Avery et al., (2005) confirmed cross-contamination throughout multiple stages of beef processing, including pre-slaughter and skinning operations in abattoirs. For instance, the transfer of *E. coli* O157:H7 in meat processing between beef tissue and high-density polyethylene board surfaces has been shown to be affected by variables such as surface roughness and beef tissue type (Flores et al., 2006).

Moreover, meat grinding, commonly used in food production has been recognized as a potential cause of cross-contamination (Flores et al., 2006). Studies indicate that *E. coli* O157:H7 can accumulate on grinder surfaces, such as the collar, and even on equipment surroundings, contaminating the final product (Flores et al., 2006; Flores & Tamplin, 2002). Bowl cutters in meat processing can harbor pathogens, particularly in areas with meat residue buildup (Flores & Tamplin, 2002).

1.16 Project Overview

This project aimed to develop and evaluate a method to control the number of biofilm-producing *E. coli* in food processing environments in Canada. We used genetically varied *E. coli* STEC strains collected from diverse environments from environmental samples to beef carcasses. Generic *E. coli* isolates found in the processing environment were included in this project as STEC can attach to or integrate within these biofilms, allowing them to persist in the environment or processing plants. Isolates used in this project are outlined in Table 4. The *E. coli* were used to evaluate stress tolerance from desiccation. Salt was used to adjust relative humidity (RH) (75% and 100%) at respective temperatures (0°C and 35°C) and stainless-steel coupons were inoculated with bacterial cells to evaluate the efficacy of this method.

Table 4. Descriptions of isolates used in this project.

Dataset	Serogroup	Number of Samples
O157	O157	96
Non-O157	O103	9
	O11	4
	O121	8
	O45	10
	O26	14
Generic *E. coli*		113

CHAPTER 2

Relationship Between Desiccation Tolerance and Biofilm Formation in Shiga Toxin-Producing *Escherichia coli* [1]

[1] This chapter from section 2.1 to 2.5 inclusive, are from Javed MQ, Kovalchuk I, Yevtushenko D, Yang X, Stanford K. Relationship Between Desiccation Tolerance and Biofilm Formation in Shiga Toxin-Producing *Escherichia coli*. *Microorganisms*. 2024; 12(2):243. https://doi.org/10.3390/microorganisms12020243. Study was formal analyzed by, M.Q.J.; investigated by, M.Q.J.; resourced by, K.S. and X.Y.; writing—original draft prepared by, M.Q.J.; writing—review and edited by, M.Q.J., I.K., X.Y. and K.S.; supervised by, D.Y.; funding acquisitioned by, K.S. and X.Y.

2.1 Introduction

Foodborne contamination and the subsequent risk of foodborne illness depend on various factors, including worker practices, food storage, food preparation surfaces, waste management and food hygiene practices (Aklilu et al., 2015; Hemalata & Virupakshaiah, 2016). Pathogens such as bacteria, viruses, fungi, and parasites can contaminate food products throughout the production line, from production and manufacturing to distribution, preparation, and final consumption (Hemalata & Virupakshaiah, 2016). Among these pathogens, bacteria, particularly those belonging to the *Enterobacteriaceae* family, pose a significant threat to the food industry (Kolling et al., 2012; Zhao et al., 2014). In particular, *Salmonella* and Shiga toxin-producing *E. coli* (STEC) genera are responsible for severe foodborne infections (Majowicz et al., 2014; Wells, 2021). Foodborne infections caused by pathogens can lead to severe harm and even death, especially among immunocompromised individuals, the elderly, and young children (Bintsis, 2017). In the case of STEC, infections are particularly concerning due to global outbreaks and their pathogenicity (Majowicz et al., 2014). Cattle serve as the main reservoir for STEC, with various serotypes linked to human sickness. Pathogenic bacteria, including STEC, can attach to surfaces and form biofilms, which present a challenge in food manufacturing settings as biofilms confer extreme resistance against sanitizers, antibiotics, and other external factors (Moser et al., 2021; Ribeiro et al., 2019; Wang et al., 2016). Over 90% of bacteria exist as a biofilm on various biotic and abiotic surfaces, including stainless steel, rubber, plastic, silicon, and glass in food manufacturing settings (Ribeiro et al., 2019). The proportion of STEC strains forming biofilms is relatively low, but STEC able to form extremely strong biofilms exist (Stanford et al., 2021). Effective control methods are required to prevent the formation of biofilms, which

can lead to cross-contamination and compromise food safety (Wang et al., 2016a; Winfield & Groisman, 2003). Various chemical, physical, and mechanical methods have been explored to control biofilm formation but are either expensive or of limited efficacy in some situations (Yu et al., 2021). These methods include the use of essential oils (Burt, 2004), enzymes (Maszewska et al., 2021; Seghal Kiran et al., 2014), biosurfactants (Salisbury et al., 2021), photosensitization (Yu et al., 2021), ultrasonic waves (Bigelow et al., 2008; Oulahal-Lagsir et al., 2000; Yu et al., 2020), and electric fields (Ravikumar et al., 2019; Sabelnikov et al., 1991). Salt is widely used in the food industry due to its preservative and antibacterial properties (Desmond, 2006). It disrupts the osmotic balance within bacterial cells, leading to osmotic dehydration when bacterial cells are immersed in hypertonic solutions (Csonka, 1989). This dehydration process can partially dewater the cells and impact their cellular components and their survival (Csonka, 1989). The effect of salt on biofilm formation and bacterial survival was judged to be of particular interest (Rahman, 2007), but to date there have been few studies evaluating desiccation for the control of biofilms. Numerous studies have reported the prolonged survival of *E. coli* O157:H7 on stainless steel surfaces, even at low temperatures (Maule, 2000; Visvalingam et al., 2017; Wilks et al., 2005). As stainless-steel surfaces are commonly used by food processors, this demonstrates the ability of STEC to persist and increases the risk of cross-contamination from contaminated surfaces to food items. By examining the resistance of *E. coli* isolates to desiccation and temperature variations, this study aimed to enhance our understanding of biofilm control strategies and the factors influencing *E. coli* survival and cross-contamination risks in food processing environments.

2.2 Materials and Methods

2.2.1. Selection of Isolates

Based on the ability of *E. coli* to form biofilm determined in a previous study (Stanford et al., 2021), a total of 254 strains were selected from both generic *E. coli* and STEC (Table 4). Briefly, over-night cultures were diluted by combining 50 µL with 5 mL of fresh Luria-Betani medium (LB, Oxoid Ltd., Basingstoke, Hampshire, UK). A 160 µL portion of the diluted inoculum was added to duplicate wells of a round-bottom 96-well microtiter plate. Each plate included duplicate blank wells with LB medium as a negative control, and positive controls featured a known strong biofilm-forming isolate (O121:H23). After incubation at 15°C for 4 d, microplate absorbance at 570 nm was measured using a microplate reader and biofilm formation was categorized as described in Table 5 The *E. coli* was originally isolated from cattle and their environment, cattle carcasses, or processing equipment as described in a previous study (Zhang et al., 2020). The STEC isolates were balanced as much as possible across different biofilm-forming classes.

Table 5. Biofilm-forming classes of *E. coli* isolates as determined by optical density (OD).

Biofilm Class [1]	**Generic *E. coli***	**STEC** [2]
0, non-biofilm former	45	92
1, weak	4	4
2, moderate	2	20
3, strong	4	10
4, very strong	10	0
5, extremely strong	48	2

[1] Biofilm-forming class is as follows: non-biofilm former, x < ODc; weak < ODc < x < 2 x ODc; moderate, 2 x ODc < x < 4 x ODc; strong, 4 x ODc < x < 8 x ODc; very strong biofilm formers, 8 x ODc < x < 16 x ODc; and extremely strong, 16 x ODc < x. [2] Shiga toxin-producing *E. coli* isolates. x = OD of two independent replicates for each isolate. ODc = three times the standard deviation of OD of negative control plus average OD of negative control.

2.2.2. Preparation of Humidity Tubes

Based on methodology from a previous study (Visvalingam & Holley, 2013), salt

(NaCl; Sigma Aldrich, St. Lou-is, MO, USA) was dissolved in distilled water to achieve RH 75% (375 g/1 L), while distilled water alone was used for RH 100%. The solutions were autoclaved and dispensed into 50 mL centrifuge tubes, with each tube containing 800 µL of the respective solution. To ensure accuracy and replicate the experiment, four tubes were prepared for each RH level (75% and 100%). All replicate tubes were processed side by side at the same time on the same day as described below. Within each RH, duplicate tubes were prepared for each equilibration temperature, with tubes incubated at 0°C or 35°C for a period of 3 weeks.

2.2.3. Coupon Preparation

Food-grade stainless steel coupons (5.0 cm × 2.0 cm, grade 304, no. 4 finish) were soaked in a detergent solution (Tergazyme, Alconox Inc., New York, NY, USA) overnight. They were subsequently washed in distilled water, followed by 70% ethanol, and left to air dry, as previously described (Visvalingam & Holley, 2013). The coupons were then autoclaved and used in the subsequent experiment.

2.2.4. Bacterial Culture Conditions

For revitalization, the bacterial culture was inoculated from glycerol stocks onto MacConkey agar plates (Dalynn, Calgary, AB, Canada) and incubated at 35°C for 24 h. Subsequently, a single colony was selected for subculture in 10 mL of ½ strength brain heart infusion broth (Oxoid). The culture was placed in a shaking incubator at 35°C and 80 rpm for 16–18 h, resulting in a bacterial suspension with a concentration of 5–6 log CFU/cm^2, which was then used for inoculating the coupons. These culture conditions were the same as used in a previous study (Zhang et al., 2020) for the same strains of *E. coli* enumerated on

MacConkey agar.

2.2.5. Inoculation and Incubation of Stainless-Steel Coupons

The coupons were inoculated by adding 50 µL of bacterial culture to one half of the coupons in individual sterile petri dishes. The petri dishes were left in a biosafety cabinet for 10 to 15 min to allow excess moisture to be absorbed. Subsequently, the equilibrating tubes were opened one at a time to prevent contamination and loss of moisture. Using sterile forceps, the inoculated coupons were carefully placed in the tubes, ensuring they did not contact the liquid, and the lids were tightly sealed. The tubes were then incubated at the same temperatures used for equilibration (0°C or 35°C) for a duration of approximately 72 h.

2.2.6. Assessment of Bacterial Counts

Freshly prepared 50 mL centrifuge tubes containing 30 mL 0.1% (w/v) pH 7.2 peptone water (Oxoid) were utilized, and the inoculated coupons with bacterial suspension were added to the tubes. Glass beads (0.5 mm) were autoclaved and pre-weighed, and 3 g was added to the tubes prior to vortexing the tubes at maximum speed for 1 min. For each sample, 10-fold serial dilutions were performed, and 1 mL of culture was filtered through a 0.45 µm membrane filter (Millipore S-Pak Type HA, 47 mm gridded, Millipore Sigma, Oakville, ON, Canada) using vacuum filtration prior to plating the filter on Lactose Monensin Glucuronate Agar (LMG Agar, Oxoid), a selective media for *E. coli*. The agar plates were incubated at 35°C for 20–24 h, and the resulting blue colonies were counted. These plates served as the control for bacterial survival without exposure to any stress environment. The same procedures were used to assess bacterial numbers on the coupons incubated for 72 h at either 0 or 35°C and these were compared to the controls.

2.2.7. DNA Extraction and PCR Confirmation

To confirm colonies were *E. coli*, DNA was extracted by lysing cells using TE buffer as previously described (Tsai et al., 1993). The amplification of bacterial DNA through PCR was performed utilizing a set of primers for the *uidA* gene (Table 6) (Bej et al., 1991), which resulted in the amplification of DNA fragments comprising 166 base pairs. Cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles, and each cycle consisted of a 30s denaturation step at 95°C, a 60s annealing step at 60°C, and an extension period at 50°C for one minute. The amplified DNA products were made visible using the QI Axcel system (QI Axcel Advanced, Qiagen, Toronto, ON, Canada). For all PCR reactions, a strain of *E. coli* O157:H7, EDL933, was used as a positive control, while a strain of *Salmonella enterica* was used as a negative control.

Table 6. Forward and reverse primers for *uidA* gene for generic PCR amplification of *E. coli* STEC.

Primer Set	Sequence
uidA	F: 5′ TGGTAATTACCGACGAAAACGGC 3′
	R: 5′ ACGCGTGGTTACAGTCTTGCG 3′

2.2.8. Statistical Analysis

Statistical analyses were performed using SAS Version 9.4 (SAS Institute Inc., Cary, NC, USA) in a mixed effects model comparison. Fixed effects included temperature, humidity, and biofilm-forming class in a factorial design. Percentage reduction was the response variable and was calculated by following equation:

$$\text{Percentage reduction} = 1 - \left(\frac{\text{CFU Treatment}}{\text{CFU Control}}\right) \times 100$$

The normality of the response variable was confirmed using Proc Univariate, with *p*

> 0.05 for each test of normality employed. Additionally, to compare the percentage reduction between STEC and generic *E. coli*, we employed RStudio (R Core Team, 2023 https://www.R-project.org, accessed on 10 January 2024). In this analysis, we performed a t-test to determine 95% confidence intervals using a Bonferroni correction to avoid inflating Type 1 error. Trends were determined if $0.05 < p < 0.1$, with significance at $p < 0.05$. The findings were visually represented using ggplot within RStudio, highlighting the distinctions between STEC and generic *E. coli*, the impact of biofilm class, and the effects of different treatments.

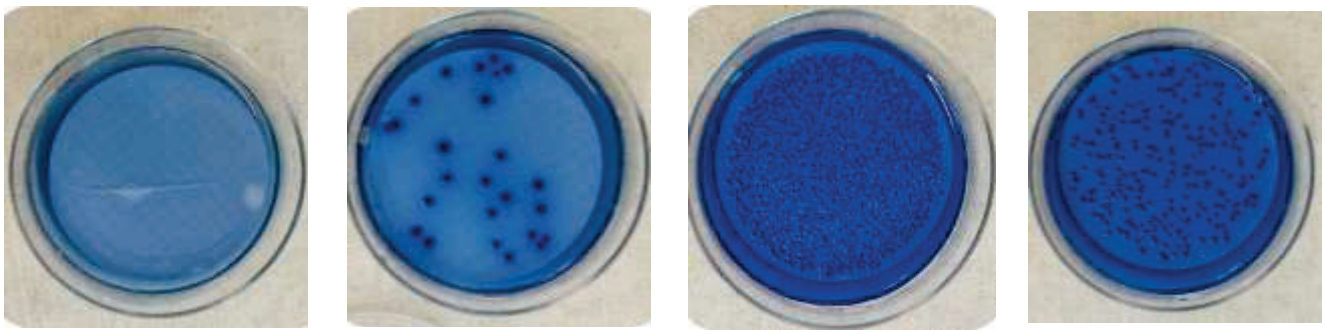
2.3 Results

2.3.1. Colony Morphology

The strains retrieved from frozen glycerol stocks were streaked on MacConkey agar plates (Dalynn), where they exhibited pink shiny textured colonies with well-defined margins. After incubation on LMG agar, colonies that lacked the expected blue coloration (Figure 3c) were occasionally noted. These included colorless colonies *(*Figure 3a) and partially blue-colored colonies (Figure 3b). Regardless of these variations in morphology, PCR amplification with *uidA* gene primers confirmed all as *E. coli*.

(a) **(b)** **(c)**

Figure 3. Unexpected colony morphology after growth of *E. coli* on LMG agar after 72 h including (a) entirely colorless colonies and (b) partial blue colonies in comparison to (c) expected blue colonies.



(**a**) (**b**) (**c**) (**d**)

Figure 4. Growth of *E. coli* strains on LMG agar under different conditions over 72 h: (a) lack of growth at 35°C and 75% RH, (b) reduced growth when exposed to 0 °C and 75% RH, (c) increased growth when exposed to 35°C and 100% RH, and (d) bacterial colonies from control without any treatment.

2.3.2. Effects of Biofilm-Producing Ability

We observed that the biofilm-forming class of the isolate had a notable impact on the growth of isolates at 35°C and 100% RH. Specifically, under what we expected to be optimal growth conditions, strains that were not capable of forming biofilm (class 0), moderate biofilm formers (class 2), and strong biofilm formers (class 3) had increased cell proliferation (> 100% of that of controls), while isolates capable of forming the strongest biofilms (class 4 and class 5) as well as weak biofilm formers (class 1) had reduced cell growth, < 100% of that of the controls (Figure 5). However, at 75% RH, the growth of all the strains was reduced compared to that of the controls and was not affected by the ability of the isolates to form biofilm.

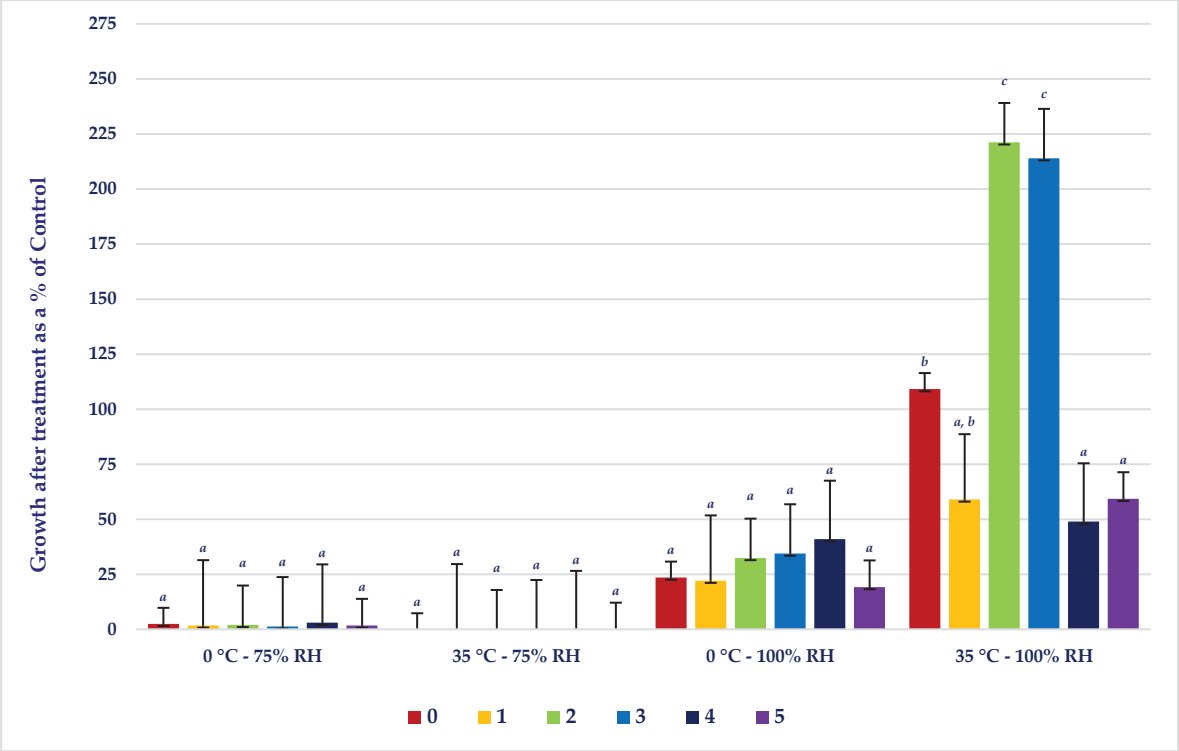


Figure 5. Influence of biofilm class on growth as a percentage of control after treatments. Comparison of desiccation tolerance between different biofilm formation categories. Biofilm classes are defined as 0 = non-biofilm former, 1 = weak biofilm, 2 = moderate biofilm, 3 = strong biofilm, 4 = very strong biofilm, and 5 = extremely strong biofilm. a,b,c Means with different superscripts within treatment combinations differ ($p < 0.05$).

2.3.3. Effects of Desiccation on STEC as Compared to Generic E. coli.

In our study, we compared bacterial reductions between STEC and generic *E. coli* across four different conditions (0°C + 75% RH, 35°C + 75% RH, 0°C + 100% RH, 35°C + 100% RH). We found no difference ($p = 0.36$) in reduction between the STEC and generic isolates at 75% RH and 0°C. Similarly, at 35°C and 75% RH, the STEC and generic *E. coli* reductions were almost identical ($p = 0.5$). However, at 0°C and 100% RH, the STEC exhibited a trend ($p = 0.1$) to a lower reduction compared to the generic *E. coli* of approximately 5.05%. Under optimal conditions, i.e., 35°C and 100% RH, the STEC isolates had a lower reduction compared to the generic isolates of approximately 85.82% ($p < 0.001$; Figure 6).

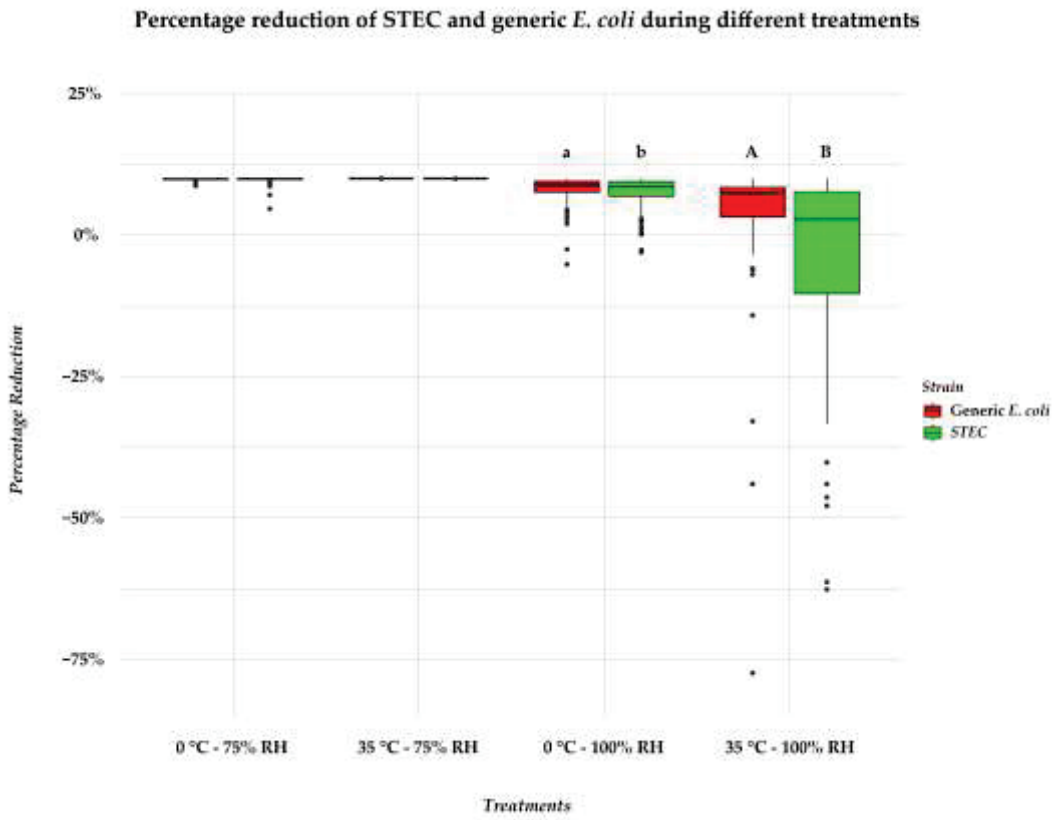


Figure 6. Box plot representation of percentage reduction values for various treatments in biofilm formation. The data are categorized by STEC and generic strains, denoted by different colors and each point indicates one isolate. The box plot illustrates the distribution of percentage reduction values within each treatment, providing insights into the variability and central tendencies of the data. Means within treatment combinations with different superscripts differ. a, b Generic and STEC strains tended to differ in percentage reduction ($p < 0.1$). A, B generic and STEC strains differed in percentage reduction ($p < 0.001$). Negative percentage reductions are indicative of cell growth.

2.4 Discussion

2.4.1. Atypical Colony Morphology

During our study, several *E. coli* isolates had colonies with unexpected morphologies when cultured on LMG agar after exposure to desiccation stress. Initially, the unexpected differences in colony appearance raised concerns of potential contamination. However, we later confirmed these colonies as *E. coli* through PCR amplification. LMG agar contains lactose as a carbon source and the lac operon is one of the best-known gene regulatory

circuits, exemplifying how bacteria adapt their metabolism to nutritional conditions (Pinto et al., 2021). Stressors that disrupt the lac operon could have led to the changes in colony color. Fluctuations in temperature can impact lactose utilization by impacting the enzymatic activity of β-galactosidase as demonstrated by Fujikawa & Akimoto (2011), where *Pantoea agglomerans* could produce blue pigment only at temperatures of ≥10°C. Also, the activity and stability of enzymes involved in pigment synthesis are directly affected by the pH of the growth media. A pH fluctuation can be induced by metabolite accumulation, nutrition intake, oxygen availability, and organic acid outflow (Fujikawa & Akimoto, 2011). The color shift by several isolates in our study was potentially caused by stress-induced gene regulation, genetic variability, and/or adaptive response.

2.4.2. Effects of Temperature and Relative Humidity

Pathogenic bacteria often encounter various abiotic stressors, such as drying, temperature fluctuations, oxidative conditions, pH changes, and osmotic pressures, among others (Wang et al., 2016). These stress factors exert selective pressures on the resilience and virulence of these bacteria, leading to immediate effects on shorter timescales and evolutionary changes over longer periods (Brouwer et al., 2019). Numerous investigations have explored the impact of these abiotic stresses on bacterial characteristics (Boor, 2006; Ezraty et al., 2017; Guan et al., 2017; Mihaljevic et al., 2007; Pinto et al., 2014). The ability of bacteria to withstand desiccation varies among different species. In general, gram-positive bacteria exhibit greater tolerance to dry conditions compared to gram-negative bacteria (de Goffau et al., 2009; Janning & in't Veld, 1994; Kramer et al., 2006). This study revealed that, even though the numbers of *E. coli* decreased on steel surfaces under dry conditions (75% relative humidity) or in the cold (0°C), certain cells persisted, while others exhibited robust

growth under the conditions of 35°C and 100% RH for over 72 h. Desiccation tolerance has been previously investigated in *E. coli*, alongside three other bacterial cultures (Suehr et al., 2020). These researchers found that *E. coli* O157:H7 displayed the lowest resistance to desiccation compared to all the other strains. The greatest reduction in O157:H7 viability was observed when it was subjected to 24 h of desiccation at a relative humidity of 33% and pH levels of 4, 5 and 7 (Suehr et al., 2020). Furthermore, Hwang et al. (2009) assessed the reduction in *E. coli* O157:H7 when exposed to temperatures of 22°C and relative humidity ranging from 80% to 85% for a period of 3 to 7 d. These researchers observed a reduction in bacterial count between 0 and 3.5 $\log^{10}$ CFU/g in sausages (Hwang et al., 2009). This finding is relevant to our current study because it underscores the relationship between lower relative humidity and a decrease in bacterial count.

Nissen and Holck (1998) reported that *E. coli* O157:H7 in dry sausages, which had a pH of 4.8 and water activity (aw) 0.89 exhibited greater inactivation when stored at 20°C compared to 4°C. Similarly, Chikthimmah and Knabel (2001) revealed that bologna inoculated with 7.5 $\log^{10}$ CFU of *E. coli* O157:H7, decreased contamination more effectively when stored at 13°C rather than 3.6°C. Interestingly, our study contradicts these, as we observed better reductions in bacterial counts regardless of temperature when the relative humidity was lower and generally reduced growth at 0°C as compared to 35°C. Desiccation stresses likely differ in a food matrix as compared to the stainless steel coupons used in the present study and differences among studies could be result of variations in the food matrix, pH, water activity, and strain-specific characteristics of *E. coli* O157:H7. The matrix where evaluations occur undoubtedly affects experimental results. *E. coli* grows rapidly in soil temperatures above 30°C but cells also have a higher mortality rate in warmer conditions

(>30°C) compared to colder temperatures (<15°C) (Ishii et al., 2006, 2010). Another study indicated that *E. coli* can endure prolonged periods at temperatures lower than those typically found in host organisms (Whitman et al., 2003) but in our study both the low and optimal temperatures could reduce bacterial growth provided relative humidity was also reduced.

2.4.3. Effects of Biofilm-Forming Class

As all the strains during the current study showed reduced growth compared to the controls after incubation at 75% RH, there were no significant differences in desiccation sensitivity among the classes of biofilm-formers. However, at 35°C and 100% relative humidity, the extent to which the isolates were able to form biofilm influenced bacterial growth. Possibly, the initial growth of the most extreme biofilm formers was rapid and used the nutrients available, resulting in cell death during the 72-h incubation. Previous studies (Coleri Cihan et al., 2017; Koerdt et al., 2010) determined that biofilm formation is most rapid at higher temperatures and the biofilm composition changes at low temperatures. When *E. coli* was cultivated in culture media with 0% or 1% NaCl, biofilm formation was detected, although the presence of 3.5% or 5% NaCl completely inhibited the development of the biofilm (Li et al., 2021). Other investigations support the notion that osmotic stress can induce biofilm production in various microorganisms, including *Staphylococcus epidermidis*, *Clostridium ljungdahlii*, and *Candida albicans* (Ferreira et al., 2019; Pemmaraju et al., 2016; Philips et al., 2017). Perhaps extreme biofilm producers are more sensitive to osmotic stress than other *E. coli* strains. Weak biofilm formers (class 1) also showed reduced growth compared to the controls at 35°C and 100% RH, possibly due to strain-related variation. The factors promoting biofilm formation in *E. coli* are poorly understood and are worthy of further study.

Another critical factor to consider pertains to oxygen availability. Changes in temperature and humidity can impact the levels of dissolved oxygen within the environment and a previous study hypothesized that elevated temperature at low oxygen availability increases biofilm formation (Kent et al., 2018). Like many other bacterial species, *E. coli* adapts its growth behavior in response to oxygen availability. As the tubes were sealed during the 72-h incubation, the initial rapid growth of the extreme biofilm formers may have reduced oxygen levels and impeded overall bacterial growth (Kent et al., 2018). Moreover, bacteria, including *E. coli*, employ QS and signaling molecules to communicate and coordinate behaviors like biofilm formation. Previous studies (Bhargava et al., 2012; Sun et al., 2014) determined that autoinducer 1 and autoinducer 2 are related to biofilm formation and regulation, respectively, in extreme environments in *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Bifidobacterium longum*. Temperature and humidity can potentially affect the production and reception of these signaling molecules, subsequently impacting the timing and intensity of biofilm development and cellular survival (Roy et al., 2022). These relationships reinforce the need to consider multiple elements when devising strategies to regulate biofilm formation, with potential implications in various fields, such as healthcare and food safety.

2.4.4. Desiccation on STEC vs. Generic E. coli

The survival rates of the STEC strains were equal to those of the generic *E. coli* in our study under 75% RH. This observation contrasts with earlier research, which had indicated lower survival rates for non-pathogenic *E. coli*. during desiccation (Hiramatsu et al., 2005) but aligned with another study using similar methodology on stainless steel coupons where complete inactivation was observed in generic *E. coli* and O157 when exposed to 75% RH and 35°C (Visvalingam et al., 2017). Despite not being widely considered resistant to

desiccation, *E. coli* O157:H7 has demonstrated greater survival abilities compared to generic *E. coli* during drying (Deng, 1998; Keene, 1997; Ryu et al., 1999). The ability of STEC to survive in dry foods has led to outbreaks caused by STEC contamination since the mid-1990s (Ryu et al., 1999). The improved growth at optimal conditions (35°C and 100% RH) for STEC noted in the present study was likely due to fewer STEC strains being able to form extremely strong biofilms compared to generic *E. coli*, with only 2 STEC extreme biofilm formers vs. 48 of the generic *E. coli*.

2.4.5. Is Desiccation a Potentially Useful Antimicrobial Hurdle for Beef Slaughter Plants?

Common advice to the food industry is to keep the production environment less humid (75% RH) to prevent bacterial growth (Møretrø et al., 2010). However, contrary to earlier findings of decreased survival at 85% RH and greater survival of *E. coli* at RH < 85% (Møretrø et al., 2010), our study observed better bacterial inhibition at 75% as compared to 100% RH. Other studies evaluated desiccation in dry foods such as confectionary products and chocolates (Hiramatsu et al., 2005) as opposed to our studies with stainless steel coupons (Heukelekian & Heller, 1940). An abundance of organic residues in desiccated food (Kuda et al., 2016), and a protective matrix in food such as sugar crystals (Carrascosa et al., 2021; Iñiguez-Moreno et al., 2019) could be possible reasons for improved bacterial survival in dry foods as compared to stainless steel under a desiccated environment.

While the meat industry typically operates at around 80% RH (Mettler & Carpentier, 1998), intermittent periods of higher humidity during cleaning routines could contribute to microbial survival. Even though this study indicated that *E. coli* survival was reduced at 75% RH after 72 h, maintaining such precise humidity levels would likely be impractical for the food industry. A more feasible approach would be to limit periods of elevated humidity at

temperatures > 0°C, as these conditions could enhance STEC survival, growth, and biofilm formation. Also, the environment of meat processing facilities would not likely have the same restrictive effect on extreme biofilm formers seen in the present study as nutrients and oxygen would both be abundant.

Previous research indicated that STEC survival rates in food were higher in dry conditions (aw 0.5 to 0.6) at 4–15°C compared to 20–35°C (Roy et al., 2021; Wilks et al., 2005; Williams et al., 2005), a trend shared by other bacteria (Kramer et al., 2006). In our study, survival was better at 35°C compared to 0°C, with an average of 24% more growth at 35°C and 100% RH, which is likely reflective of differences in the survival of *E. coli* in food as compared to stainless steel. This study's findings indicate desiccation at 75% RH was effective for growth inhibition regardless of temperature, although inhibition was most pronounced at 0°C. Accordingly, desiccation may be a valuable strategy for mitigating the prevalence of enteric pathogens on surfaces within the context of beef processing. The utility of NaCl sprays, independently and in tandem with more aggressive compounds such as sodium hypochlorite, n-alkyl dimethyl ethyl benzyl ammonium chlorides, and peroxyacetic acid is worthy of additional study.

2.5 Conclusion

Our study explored the impact of desiccation stress on E. coli, uncovering unexpected results. Atypical colony morphologies initially raised concerns, but PCR confirmation verified them as *E. coli*. Our research showed that 75% RH yields better bacterial inhibition than 100% RH, in contrast to some other studies evaluating desiccation effects in food matrixes. The investigation showed that biofilm formation involves complex dynamics, with bacterial

growth being influenced by temperature and humidity. Survival of the STEC and generic *E. coli* was equivalent at 75% RH but differed most markedly at 35°C and 100% RH, likely due to the reduced biofilm- forming ability of STEC. Overall, our research indicates that desiccation at 75% humidity might be a promising approach for mitigating enteric pathogens on surfaces in beef processing. However, additional studies are necessary to determine when to apply salt sprays in conjunction with traditional sanitizers.

CHAPTER 3

Final Conclusions and Future Directions

3.1 Thesis Summary

The objective of this study was to assess the efficacy of desiccation stress in eradicating bacterial contamination, with a focus on biofilm-forming generic *E. coli* and STEC stainless steel surfaces as would be found in beef processing plants. Using salt and water, we manipulated relative humidity to induce different levels of stress on the bacteria. Beef processing employs different techniques to address bacterial contamination, each having its own advantages and disadvantages. We used salt and water to manipulate relative humidity and examine STEC strains' tolerance under different environmental conditions in our study. Our experiments involving various humidity and temperature conditions revealed that a relative humidity level of 75% was the most successful in eliminating STEC from surfaces, regardless of temperature. We also observed bacterial proliferation at 100% RH compared to controls, particularly at 35°C. Based on our findings, the integration of salt or saltwater solutions has the potential to reduce bacterial contamination in beef processing plants. However, it can be challenging to maintain optimal humidity levels during processing. Nonetheless, in real-world scenarios, a practical solution could involve alternating between optimized and general humidity levels through periodic adjustments.

Furthermore, by incorporating salt or saltwater solutions into washes for hide-on carcasses, hides can be cleansed, reducing bacterial contamination and promoting hygiene. Saltwater sprays can be strategically used on the processing line to target vulnerable areas and decrease bacterial loads due to biofilm formation. The use of salt chambers or enclosures in processing plants and precise treatment of equipment and surfaces, may improve eradication of bacterial contamination. Moreover, the hygroscopic properties of salt can naturally control humidity levels in processing environments, providing a sustainable and affordable solution.

Nevertheless, it is crucial to consider the possible negative impacts of using salt and saltwater. Corrosion in metal surfaces can be accelerated by saltwater, which can impede sanitation by promoting bacteria growth and residue accumulation. Moreover, the disposal of saltwater solutions can harm local ecosystems and have environmental repercussions. Factors to consider include sensory changes in meat products and health concerns tied to high salt intake. Adhering to proper protocols is vital in beef processing to meet food safety regulations and manage risks linked to salt and saltwater use. Accordingly, use of salt-water sprays or washes would need to be minimized and used strategically for maximum impact.

3.2 Final Conclusions

To conclude, our research emphasizes the efficiency of utilizing desiccation stress, facilitated by salt and water, as an effective approach to combat bacterial contamination in beef processing plants. Through manipulation of relative humidity levels, we observed notable declines in biofilm-forming *E. coli* strains, including STEC, on stainless steel surfaces. Our research demonstrates how salt and saltwater solutions effectively control humidity, reducing bacterial growth. Furthermore, incorporating salt or saltwater solutions at different points in beef processing, such as cattle washing, surface disinfection, and humidity control, offers a versatile approach to improving food safety protocols. Although there are challenges such as equipment deterioration and environmental consequences from use of salt water, these concerns can be mitigated by strategic implementation of desiccation. Future research should focus on the practicality and scalability of salt-based methods in beef processing plants. It is crucial to continuously refine salt concentrations, application techniques, and monitoring mechanisms to maximize effectiveness and minimize negative impacts. By adding to current knowledge on bacterial control methods in food processing, our research highlights the value

of salt and water-based approaches in enhancing food safety in beef processing.

3.3 Future Directions

Comprehending the associated risks of biofilm contamination, especially in relation to STEC in beef, requires additional research. Ongoing research highlights the prevalence of STEC in beef contamination via biofilms, underscoring the importance of comprehending biofilm formation. To gain a comprehensive understanding of biofilm development, future research should use scanning electron microscopy to differentiate microcolonies from fully mature biofilms. Furthermore, the progress in biofilm monitoring necessitates the creation of cost-effective techniques for sampling and quantifying bacteria in biofilms more efficiently.

To improve the reproducibility of biofilm studies, future research could use STEC-contaminated beef as the initial biofilm seed, providing a more accurate representation of natural biofilm development. The study highlights how desiccation may decrease STEC transmission to beef, indicating the possibility of humidity control to prevent biofilm formation and lower STEC contamination risks. Additional investigation is required to assess the practicality of implementing these measures at various stages of the slaughter process.

In the future, research should focus on advanced imaging techniques, improved sampling methods, biofilm cultivation on beef, and directly assessing desiccation as a control measure in meat processing. Through these investigations, a more profound comprehension of biofilm contamination of beef can be attained, resulting in improved management of STEC biofilm risks.

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APPENDIX

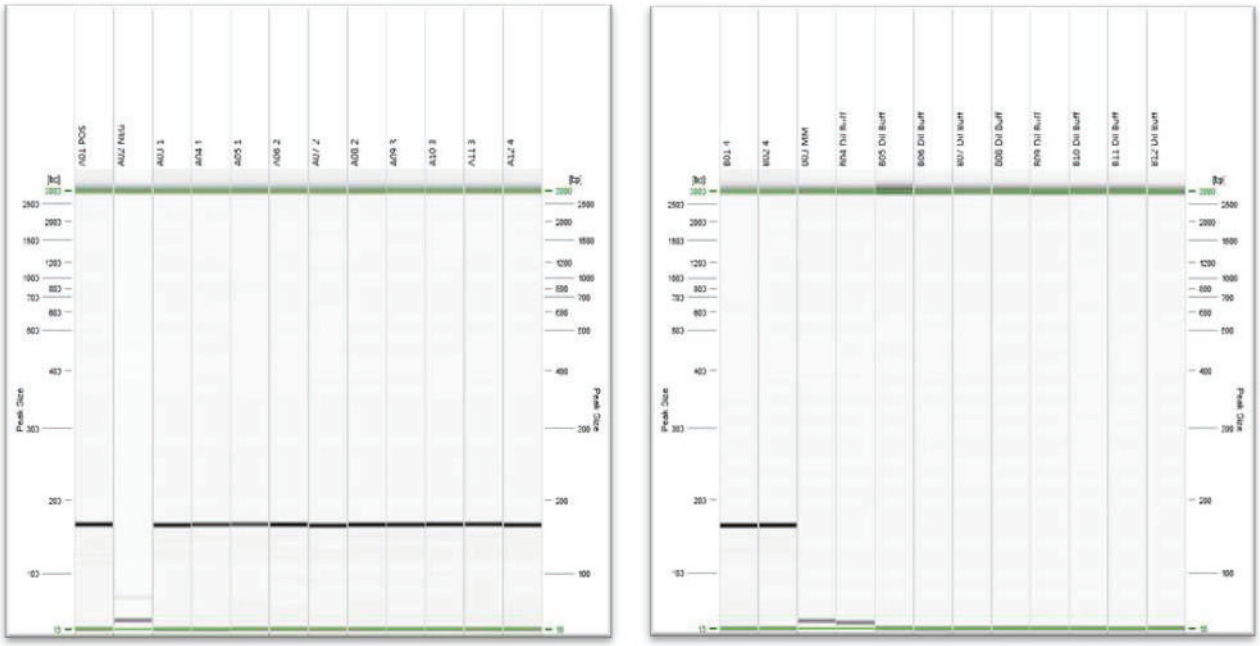

Figure S1. QI Axcel showing PCR amplified products from bacterial DNA. Lanes: 1 ladder, 2: PCR water, 3-4: positive control (EDL933 DNA), 5-6: white colonies, 7-8: white colonies along with some blue ones, 9-10: blue colonies along with white ones, 11-12: partial colorless colonies, and 13-14: negative control (master mix).