Shiga Toxigenic *Escherichia coli* Biofilm Persistence in Beef Processing Environments and Contamination of Beef

by

Matthew Wells

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of requirements of the degree of

MASTER OF SCIENCE

Department of Food and Human Nutritional Sciences

University of Manitoba

Winnipeg

ABSTRACT

Shiga Toxigenic *Escherichia coli* (STEC) are a group of pathogenic bacteria of high importance to food regulatory authorities, consumers, and processors due to the public health risk they pose. The objective of this research was to quantify the effect of time, temperature, and moisture on the development of STEC biofilms and subsequent contamination of beef tissues. Initially, all 13 STEC strains were screened for biofilm formation at different temperatures and growth times using crystal violet to characterize strains as weak, moderate, strong or non-biofilm forming. However, only one weak and one strong biofilm forming strain were selected for further testing. Contamination of beef was tested by growing biofilms at different temperatures (2, 10 and 25ºC) and growth times (2, 4 and 6 days), followed by an aging period (2, 4, 6, 30 days) at two moisture levels (wet or dry) before testing transfer to beef tissue (lean or adipose). An additional set of coupons were additionally prepared under the same conditions and enumerated directly. The beef was centered on the coupon, followed by a 50g weight placed on the meat to simulate biofilm-to-beef transfer. The genome sequences of the two tested strains were annotated using RASTtk and screened for genes related to biofilm formation. Of the 13 tested STEC strains, strong biofilms were only detected at 25 ºC after 4 days. Strain O157:H7 R5O8 formed a strong biofilm, while O26-2 only formed a weak biofilm at 25ºC. Tissue type was not found to affect contamination of beef from biofilms on the stainless-steel coupons ($p > 0.05$). While transfer from wet biofilms to beef reached 3.2 Log (CFU/cm^2) with transfer continuously decreasing over a 30-day period. Contamination from dry biofilms was minimal, as less than 1 Log (CFU/cm^2) was transferred to beef or able to be enumerated directly from the coupons. Of the genes screened for only *(tolA)* was found to differ between O26-2 and O157:H7 R5O8. This research shows that biofilms formed by STEC can contaminate beef after prolonged periods of nutrient

deprivation and desiccation. Emphasizing the need for suitable cleaning and sanitization protocols which target biofilm formation.

ACKNOWLEDGEMENTS

I would like to thank friends and family for their support in finishing my thesis I would not be where I am without them. I would like to extend special thanks to my dear friend Dr. Gerardo Medina for his tireless support helping me till 1 am in the lab on multiple occasions, I would not have completed much of my work without him. Additionally, my good friend Yuchen Nan who brought was a source of joy throughout my work. I would also like to thank Dr. Franklin Apea-Bah for always being around the building for an uplifting conversation. Dr. Argenis Rodas-Gonzalez for showing me the wonders and joys of statistics, aiding the development of a critical mindset when it comes to experimental design. Dr. Charles Grant for supporting my new ideas and always being open for a stimulating discussion of them.

I would like to thank both of my parents John and Tamara Wells for their constant support throughout this experience. Finally, I would like to thank Taylor Davedow for her much-needed wisdom available at any moment.

Finally, I would like to thank my advisors Dr. Claudia Narvaez of the Food Science department at the University of Manitoba for always pushing me to do better. Along with, Dr. Tim McAllister of Agriculture and Agri-Food Canada at the Lethbridge Research and Development Center for always providing me with new opportunities to present my research.

DEDICATION

To my girlfriend Taylor Davedow, who was by my side throughout my entire degree.

Along with those who have suffered due to STEC, perhaps one-day we can bring an end to food borne illness.

Table of Contents

Table of Abbreviations:

Abbreviation	Definition
BPW	Buffered Peptone Water
CFIA	Canadian Food Inspection Agency
DAEC	Diffusely Adherent *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EPS	Extrapolymeric Substances
FSIS	Food Safety and Inspection Service
GMP	Good Manufacturing Practices
HC	Haemorrhagic Colitis
HDPE	High Density Polyethylene
HEP	High Event Period
mTSB	Modified Tryptone Soya Broth
QAC	Quaternary Ammonia Compounds
QMRA	Quantitative Microbial Risk Assessment
STEC	Shiga Toxigenic *Escherichia coli*
USDA	United States Department of Agriculture
VTEC	Verotoxigenic *Escherichia coli*

List of Tables:

List of Figures:

Introduction:

Persistent contamination of beef by Shiga toxigenic *Escherichia coli* (STEC) is a major problem affecting the Canadian and American Beef Industry, with biofilm formation by STEC speculated as a potential mechanism for beef contamination (Winfield & Groisman, 2003). In 2018 alone, there were 7 recalls related to *E. coli* O157:H7 in beef products in Canada (Canadian Food Inspection Agency, 2019b), and 9 related to STEC in the United States (United States Department of Agriculture, 2018). While beef recalls are costly to individual meat processors, they negatively impact the beef market and consumer perceptions for the safety of beef (Cranfield, 2013). Estimates relating to the economic impact of STEC on the beef industry generated using Canadian beef sales data gathered between 2008 and 2010 suggest that each additional beef recall results in a loss of 8 to 67 million dollars to beef producers in Canada, with recalls in the U.S.A also affecting Canadian beef prices (Cranfield, 2013). Prior to 2013, the 2012 XL foods outbreak was a particularly noteworthy outbreak resulting in 18 Canadians falling ill due to infection by *E. coli* O157:H7, even after the recall of affected meat products had been initiated (Lewis, Corriveau, & Usborne, 2013). The XL Foods recall impacted over 20 countries and is estimated to have cost the Canadian beef industry approximately 16 to 27 million dollars. Additionally, the XL Foods plant was closed as a result of the outbreak, which at the time represented 35% of Canada's processing capacity (Lewis et al., 2013). The public has also become increasingly concerned about the safety of Canadian beef, with 50% of Canadians surveyed believing that O157:H7 illnesses were on the rise despite STEC related infections per capita declining from 4.5 infections per 100,000 people averaged between 1993 to 1999 to 1.8 infections per 100,000 people averaged between 2009 to 2019 (Gill, 2018; Lewis et al., 2013).

As STEC can causes serious illness in humans, beef producers and meat processors have been proactive in developing/validating controls to prevent STEC contamination of beef. Controls for STEC within the beef industry are developed using a multi-hurdle approach, with each process reducing the risk of STEC contamination. Implemented hurdles are assessed for effectiveness and approved by regulating agencies like CFIA/FSIS and incorporated into Hazard Analysis Critical Control Points (HACCP) plans. The hurdles employed for prevention of STEC appear to be effective, as evidenced by the decrease in beef associated STEC illnesses (Gill, 2018). Despite the reduction in human cases of O157 in Canada, it is still unclear which interventions are responsible for reducing levels of STEC illnesses (4.5 to 1.8 infections per 100, 000) (Gill, 2018). As increases in sampling can identify STEC contaminated beef and subsequently preventing it from entering the market, it does not prevent the initial contamination of beef. It is likely that the reduction in STEC illnesses reflects the multiple mitigation measures that have been introduced throughout the beef production chain.

Biofilms which are the sessile communities of microorganisms, in which they embed themselves in an extracellular matrix of substances that protect the community from sanitizers, oxidizers, desiccation, and thermal stress (R. Wang, Bono, Kalchayanand, Shackelford, & Harhay, 2012). Biofilm formation can occur on production surfaces or foods if contaminated by bacteria capable of forming a biofilm (Ryu, Kim, Frank, & Beuchat, 2004). Biofilm formation is a sequential process starting with attachment, microcolony formation and the formation of a mature biofilm (Figure 1) (Stoodley, Sauer, Davies, & Costerton, 2002). Additionally biofilm formation has been shown to increase the survivability of *E. coli* from sanitizers increasing rates of survival by ~1 Log (CFU/g) after sanitizer exposure (Ölmez & Temur, 2010). The increased survivability of STEC in a biofilm creates a risk of sporadic cross-contamination of beef

products as bacteria are shed intermittently from biofilms (Srey, Jahid, & Ha, 2013). While surface swabbing is used to verify sanitation procedures in beef processing facilities, STEC living in biofilms may not be removed by swabbing, and thus go undetected, due to the biofilm preventing the bacteria being transferred from the surface (J. Verran, Boyd, Hall, & West, 2010). If a biofilm remains intact, the sloughing of STEC cells back into the meat processing environment can result in further contamination (Figure 1) (Srey et al., 2013).

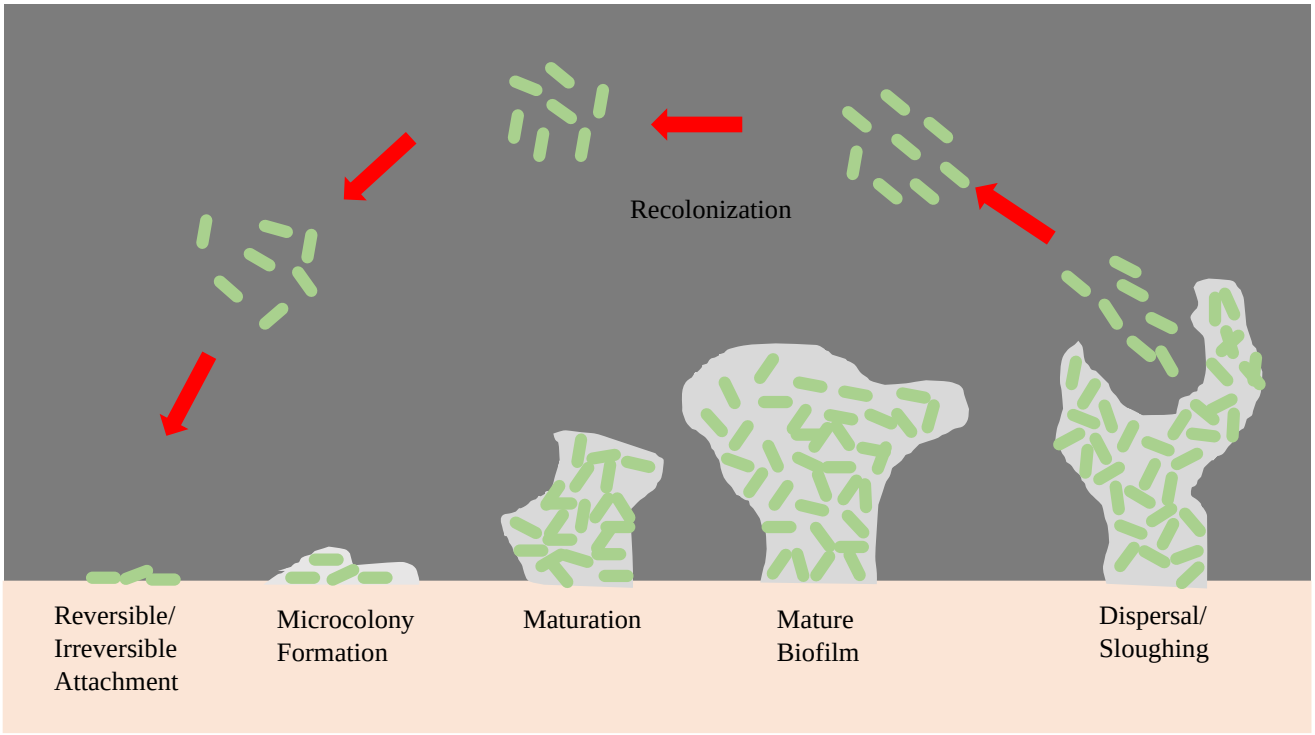


Figure 1. The biofilm lifecycle adapted from (Stoodley et al., 2002).

It is possible that placing an emphasis on the potential risk that STEC biofilms pose in contamination of beef and adopting sanitation, cleaning and surveillance procedures targeting biofilms may reduce the risk of beef being contaminated. Targeted surveillance of biofilms is a practice already adopted by producers of Ready-to-Eat foods like MapleLeaf foods, who dealt with the aftermath of a *Listeria* outbreak and developed supplementary cleaning protocols to

mitigate biofilms (Maple Leaf Foods, 2021). Strategies and practices employed by companies which have been responsible for outbreaks such as MapleLeaf foods now regularly disassemble and deep clean/sanitize work surfaces to prevent contamination from biofilms. Removal of biofilms is additionally mentioned in the Codex Alimentarius international guidance documents related to plant sanitation, which states that effective cleaning procedures should be used to prevent or remove any preformed bacterial biofilms (Codex Alimentarius Commission, 1999). Meat processors are not legally required to test for the presence of STEC biofilms, but they may choose to include biofilm sampling in their operation (Canadian Food Inspection Agency, 2019c). However, scientific data on the role of STEC biofilms in the contamination of beef are scarce.

Biofilms have also been theorized to be a possible cause of high event periods (HEPs), which are periods when STEC is detected by sampling procedures at a rate that is 5% higher than the normal detection frequency (Food Safety and Inspection Service, 2014). The elimination of high event periods in the beef industry could reduce product losses, but the causes of HEPs remain unknown. Additionally, the true cost associated with HEPs has not been fully quantified, as HEPs are determined within a processing plant and therefore require food processors to report losses related to HEPs. Yet HEPs do increase the risk of product being contaminated resulting in either discarded or reworked product, increased sampling, increased sanitation measures and if contaminated product has been released to market a recall and potential human illness. All of the aforementioned consequences of a HEP result in loss of revenue for an establishment and a potential human cost (Food Safety and Inspection Service, 2014)

Hypotheses:

The information gathered in this project will be used to help understand how environmental conditions during beef processing impact the cross-contamination of beef by STEC biofilms.

The hypotheses are:

A. **Dry biofilms will transfer more bacteria than wet biofilms**. Similar studies have observed that dry *Listeria monocytogenes* biofilms (Rodriguez & McLandsborough, 2007) and dry O157: H7 biofilms transfer more bacteria to meat than wet biofilms (Flores, Tamplin, Marmer, Phillips, & Cooke, 2006). The suspected mechanism of increased dry biofilm transfer is decreased cell-to-cell or biofilm to cell-adhesive forces within dry biofilms which may become weakened as aging of the dry biofilm is prolonged. As when a moist food item comes in contact with a dry biofilm, hydration bridges form that increase the transfer of bacteria as less shear force is required to disrupt the biofilm (Rodríguez, Autio, & Mclandsborough, 2007). Additionally, the increased growth times at higher temperatures (e.g., 25 $^{\circ}$C) will allow for increased biofilm formation resulting in an increased bacterial load in the biofilm allowing for larger levels of bacterial transfer.

B. **Transfer of STEC to adipose tissue will be higher than to lean tissue**. The reasoning behind this hypothesis is based on other studies that have observed that flagellated *E. coli* (STEC with an H antigen) have a higher rate of attachment to hydrophobic surfaces than un-flagellated *E. coli* (Friedlander, Vogel, & Aizenberg, 2015). The O antigen (lipopolysaccharide) will also aid in increasing the surface hydrophobicity of the bacteria which may result in increased adherence to adipose tissue due to Van der Waals forces (Dickson, Koohmaraie, & Hruska, 1989). Once again increased growth times at higher temperatures will allow for increased levels of bacterial transfer due to higher levels of biofilm mass allowing for more bacterial transfer.

Objectives:

The objectives of this project are as follows:

1. Characterize biofilm-forming strength of major STEC serovars *in vitro*. Serovars were provided by the USDA, strains tested were: O103-2, O103-3, O45-3, O45-4, O111-3, O111-4, O121-3, O121-4, O145-2, O145-3, O26-2, O26-3.
2. Compare the ability of dry and wet biofilms formed on stainless-steel 304b coupons to enable transfer of two STEC serovars to lean (muscle) tissue vs. adipose (fat) beef tissue.
3. Determine the effect of time and temperature and aging of both dry and wet biofilms grown on stainless steel coupons on transfer to beef tissue testing hypothesis A.
4. Determine the effect of time and temperature on STEC biofilm formation by measuring STEC transfer from stainless steel coupons to lean and adipose beef tissue to test hypothesis B.

1 Review of Literature:

1.1 Shiga Toxigenic *Escherichia coli*:

1.1.1 STEC History & Classification:

Escherichia coli O157:H7 was first identified as an enteric pathogen after an outbreak in Oregon U.S.A in 1982 where 47 individuals became ill from consuming hamburgers purchased from a McDonald's restaurant (The Marler Clark Network, 2020). In 1993, the Jack-in-the-Box outbreak occurred which at the time was considered America's most tragic foodborne outbreak, in which 700 people became ill and 4 died (The Marler Clark Network, 2008). While O157:H7 was first characterized in the McDonald's outbreak, it is believed that the first case of O157 illness occurred in 1975 when the serotype was associated with a clinical case of bloody diarrhea (Law, 2000). Researchers later identified virulence factors associated with other pathogenic serotypes of *E. coli*, in which it was observed that the verotoxin produced by STEC serovar O26, and the Shiga-toxin produced by O157 killed both Vero and HeLa cells. Both toxins were neutralized by Shiga toxin 1 antiserum (O'Brien, Lively Thomas, & Chen Melinda, 1983). Researchers also noted that STEC were capable of invading the intestinal epithelium and producing a heat-stable enterotoxin, a trait not associated with previously identified in *E. coli* (O'Brien et al., 1983).

O157 is not the only STEC serovar and there has been earlier outbreaks attributed to uncharacterized serovars. The O26:H11 serovar has been known to cause human illness for nearly 70 years with isolates associated with infection in humans dating as far back as 1952 (Ogura et al., 2017). In 1947, there were 417 cases of gastroenteritis attributed to the *E. coli* serovar O111, although the food source which caused the outbreak was never identified (Belnap & O'Donnell, 1955). Both O111 and O26 would later become part of the "Big Six", which are STEC serovars that are specifically tested for and reportable to the Food Safety and Inspection

Service (FSIS) a part of the USDA due to their likelihood in causing foodborne illness, and includes other serovars such as: O103, O45, O145 and O121 (Bertoldi, Richardson, Schneider, Kurdmongkoltham, & Schneider, 2017). However, following the 1982 O157:H7 outbreak, the distinction between enteropathogenic *E. coli* (EPEC), Verotoxigenic *E. coli* (VTEC), and Shiga-toxigenic *E. coli* (STEC) was made. Since the first description of pathogenic *E. coli*, different serotypes have been classified into separate categories based on biological characteristics and pathogenesis, including EHEC (which may also be referred to as STEC), EPEC, Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely adherent *E. coli* (DAEC) (Table 1).

Table 1. *Escherichia coli* Pathotypes and Defining Characteristics

Pathotype	Characteristics
Shiga-Toxigenic/Vero-Toxigenic *E. coli (STEC, VTEC)*	• Invade intestinal epithelium. • Can form A/E lesions • Produces Shiga toxins • Symptoms of infection can be moderate or severe. • Can lead to potential development of Hemolytic Uremic Syndrome • Causes deterioration of the intestinal membrane (Law, 2000; O'Brien et al., 1983)
Enterohaemorrhagic *E. coli (EHEC)*	• Subgroup of STEC. • Can lead to potential development of Hemolytic Uremic Syndrome (Mayer, Leibowitz, Kurosawa, & Stearns-Kurosawa, 2012) • Causes Haemorrhagic Colitis. • STEC associated with severe colonic and renal diseases renamed as EHEC (Goldwater & Bettelheim, 2012)

Enteropathogenic *E. coli (EPEC)*	• Creates attaching and effacing lesion (A/E lesion). • Destroys intestinal microvilli and integrates into host cytoskeleton. • Main symptom is diarrhea (Trabulsi, Keller, & Tardelli Gomes, 2002)
Enteroaggregative *E. coli (EAEC)*	• Subgroup of EPEC. • Like DAEC except forms a microcolony on the intestinal epithelium. • Does not form an A/E lesion. • Forms a mucus-like biofilm in the intestinal tract. • Many strains are likely to cause diarrhea (Nataro & Kaper, 1998)
Enterotoxigenic *E. coli (ETEC)*	• Main symptom is diarrhea. • Secretes either heat-stable (ST) or heat-labile (LT) toxins. • 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)*	• Infection with EIEC includes penetration into an epithelial cell followed but intracellular replication and extension into adjacent cells. • EIEC strains are genetically and biochemically related to *Shigella* spp. • Secretes enterotoxin causing diarrhea (Nataro & Kaper, 1998)
Diffusely Adherent *E. coli (DAEC)*	• Subclass of EPEC. • Characterized by a random distribution of bacteria along a cell surface. • Does not show microcolony formation within the intestine. • Commonly causes diarrhea by the production of toxins or intestinal inflammation (Servin, 2014)

The O157:H7 STEC serovar has been highly studied and a divergent evolutionary path was identified within the O157:H7 genome, using octamer-based genome scanning (Kim, Nietfeldt, & Benson, 1999). It was found that the O157:H7 genome can be split into two lineages: lineage 1 which is adapted to a human host, and lineage 2 which is adapted to a bovine host (Kim et al., 1999). It was later concluded that the majority of human illness related to O157 comes from lineage 2 strains with partial adaptations to a human host, and it is thought that these adapted O157 strains are responsible for the majority of human-related illnesses (Kim et al., 1999; Law, 2000). However the differences between lineages 1 and lineage 2 which lead to increased pathogenicity of certain lineage 2 O157 strains is unknown and is speculated to be related to uptake of novel adhesins (Law, 2000). There are other STEC serovars of interest besides O157 (non-O157 STEC) which have been identified and referred to as the "big six" which include O26, O45, O103, O121, O111, and O145 as they are the serovars most commonly associated with hospitalization of patients (Food Safety and Inspection Service, 2012). Although the lineages of O157:H7 have been characterized based on the source of isolation as being either from humans or cattle, the same has not been done for the big six STEC serovars. These serovars have been classified using a modified scheme for STEC proposed by Karmali et al. (2003), as not all STEC serovars have the same pathogenic capabilities (Karmali et al., 2003). This altered classification scheme has been denoted as the seropathotype model. With this classification, STEC strains are divided based on their capacity to cause illness and outbreaks. Strains which are likely to cause haemolytic uremic syndrome (HUS), and are commonly associated with outbreaks are denoted as seropathotype A which currently only includes O157:H7 and O157:NM, NM (non-motile) referring to serotypes lacking an H antigen or flagella (Karmali et al., 2003; Pintara et al., 2018). Seropathotype B includes the STEC serovars O26:H11, O103:H2,

O111:NM, O121:H19, O145:NM, as these serovars are less likely to cause HUS, and are not as frequently associated with outbreaks that cause human disease. Seropathotypes A and B consist of those STEC serotypes that are most relevant to human illness, with exception of the O45 serovar which remains uncharacterized. The seropathotypes listed in Table 2 include only a subset of the seropathotypes with the list ranging from A to E, with the order designating the likelihood of causing human illness (Karmali et al., 2003). Since the seropathotype classification scheme has been first introduced in 2003 modifications have been made by different regulating agencies to reflect an increases in illness attributed to STEC serovars other than O157 (Messens et al., 2015). As serotypes like O104:H4 would have previously been classified as seropathotype D due to a lack of association with outbreaks, have since been responsible for large scale outbreaks involving 3816 persons, 845 cases of HUS and 54 deaths (Messens et al., 2015). Additionally, as of 2019 the incidence of STEC illness due to non-O157 serovars nearly doubles that of illness caused by O157 serovars, with a large portion of illness being attributed to O26 serovars and other non-O157 species (National Enteric Surveillance Program, 2019). The rise in non-O157 STEC cases reflects the need to alter the current distribution of serotypes within the seropathotype model or for a new classification scheme of STEC and severity. Some STEC strains may also be designated as Enterohemorrhagic *E. coli* (EHEC), and this designation extends to all serovars capable of causing hemorrhagic colitis (HC) (Delannoy, Beutin, & Fach, 2013).

Table 2. Classification of STEC serotypes into seropathotypes.

Seropathotype	Relative Incidence	Frequency of Involvement in Outbreaks	Association with Severe Disease [a]	Serotypes
A	High	Common	Yes	O157:H7 O157:NM
B	Moderate	Uncommon	Yes	O26:H11 O103:H2 O111:NM O121:H19 O145:NM
C	Low	Rare	Yes	O91:H21 O104:H21 O113:H21
D	Low	Rare	No	Multiple
E	Nonhuman Only	NA [b]	NA	Multiple

[a] Severe illness includes either hemolytic uremic syndrome or hemorrhagic colitis.
[b] NA, non-applicable (no associated outbreaks or association with severe disease).
Note: The above table is adapted from (Karmali et al., 2003)

1.1.2 STEC Pathogenicity:

The capability of STEC to produce Shiga toxin and cause harm in humans is what separates generic *E. coli* from STEC. STEC bacteria contain multiple virulence factors like intimin (*eae*), Shiga toxins (stx) and enterohemolysins (*ehxA*), along with other virulence factors (Law, 2000). Intimin is an attaching and effacing protein produced by STEC and EPEC bacteria which allows the bacteria to attach to the intestinal epithelium, where it will form a lesion and disrupt the cytoskeleton of the host cell (Jerse, Yu, Tall, & Kaper, 1990). This protein allows for STEC to remain in the intestinal tract of the host, where it can continuously produce toxins such as Shiga-toxin causing illness. Shiga-toxins which are what defines STEC and can be divided into two groups, stx1 and stx2 with Shiga-toxin 2 being 1000 times more toxic than stx1 despite

structural similarities (Louise & Obrig, 1995). These toxins are also believed to be responsible for causing hemolytic uremic syndrome (HUS), a condition that results in acute renal failure (Louise & Obrig, 1995). The Shiga-toxins were believed to be acquired by *E. coli* through a phage vector and are thought to have originated from *Shigella dysenteriae* (Law, 2000). The enterohemolysin produced by STEC causes lysis of red blood cells and is often associated with the presence of Shiga toxins, but the role of hemolysin in pathogenicity has yet to be fully described (Fu et al., 2018).

Although STEC can cause clinical disease in humans, cattle are largely unaffected by STEC despite being the main reservoir of the pathogen. While the majority of serotypes appear asymptomatic in cattle, some serovars (such as O5:H-, O26:H11, O103:H2, O111:H8, O111:H11, O111:H- and O118:H16) have been observed to cause hemolytic uremic syndrome (HC) in calves and have been termed as bovine EHEC (Johnson et al., 1996). Why some STEC serovars cause HC in calves and others do not is still unknown, as cattle lack the receptors for Shiga toxins in their blood vessels, but some cells within the ruminant digestive tract, like mesenchymal mucosa cells do contain receptors for the stx1, which if present can reduce cell replication within the intestinal tract (Moxley & Smith, 2010). Additionally, STEC was observed to cause immunosuppression within calves leading to further colonization of STEC within the lower intestinal tract of cattle (rectum and cecum), possibly leading to HC (Moxley & Smith, 2010). Pigs can also develop STEC infections, resulting in ataxia, convulsions, and paralysis due to vascular injuries caused by Stx2 (Johnson et al., 1996).

1.2 Control of STEC in Beef:

1.2.1 Prevention of STEC in Beef Processing:

Currently, microbial hazards are mitigated in meat processing plants by the adoption of Hazard Analysis Critical Control Points (HACCP) systems to minimize the risk of microbial contamination (Canadian Food Inspection Agency, 2019a). Changes involving improvements in process hygiene such as modifying equipment by making it easier to disassemble to enhance cleaning, reducing bacterial colonization and providing improved training of personnel (Duckworth, 2013). As STEC can be found at all points of the food chain, pre-harvest and post-harvest controls are employed to reduce the risk of STEC contaminating food and reaching consumers.

Pre-harvest interventions are those which take place on farm and involve STEC exposure reduction strategies such as cleaning pens, proper biosecurity practices and experimental approaches like adding sodium chlorate to water as a mitigation strategy, developing STEC vaccines and in-feed probiotic all with the goal of reducing STEC colonization within the digestive tract. Not all pre-harvest reduction strategies have been implemented as part of standard operating procedures, but each additional intervention acts as a hurdle to reduce the risk of STEC contamination during later post-harvest processing steps (Smith, 2014; Wheeler, Kalchayanand, & Bosilevac, 2014).

To ensure food safety during post harvest processing (i.e., slaughter and processing), the meat industry employs a multi-hurdle approach in their HACCP plans to reduce the risk of STEC and other foodborne pathogens causing contamination in processing plants. These practices include proper cold chain maintenance, frequent microbiological sampling, and proper hygienic practices during slaughter which are included as part of industry sanitation standard operating

procedures (SSOP) and good manufacturing practices (GMP)(Food Safety and Inspection Service, 2017). While post-harvest microbial interventions are employed to reduce the level of STEC on beef products and include hide-on carcass washing, skinned carcass washing, proper head removal, evisceration strategies, trimming of visual contamination, steam vacuuming, spraying with lactic acid or other antimicrobial solutions, carcass pasteurization and proper chilling (Lewis et al., 2013).

1.2.2 STEC Sampling:

Regular sampling of surfaces and meat products is a frequent activity carried out by personnel within a beef processing plant. Environmental sampling occurs regularly as way of validating sanitation operations, validating new operations and to aid in surveillance of potential pathogens within the processing plant. Product sampling is additionally used as a way of verifying the safety of a food and aiding in prevention of contaminated products from entering the food chain. As product sampling is often destructive and expensive, subsamples of lots are often used to verify safety of product lots.

For the purpose of detecting O157 STEC in beef various sampling schemes have been developed with the N60 sampling scheme being used in Canada (Canadian Food Inspection Agency, 2019c). The N60 sampling protocol is targeted at detecting 95% of beef lots contaminated with STEC, and involves the collection of 60 samples from a lot (Beef Industry Food Safety Council, 2016). The N60 sampling procedure involves the collection of 60 random samples from a lot, with a lot typically being approximately 10,000 pounds of ground meat (American Meat Institute, 2009) and within Canada 60 samples is the minimum number of sub-samples required, regardless of the lot size (Canadian Food Inspection Agency, 2015). Canada currently only considers O157:H7/NM as an adulterant (Canadian Food Inspection Agency,

2015), Thus beef is only tested for O157:H7 serovar; but when beef is exported to the U.S.A it is also tested for the six STEC serovars (O145, O26, O121, O111, O45, O103). Testing for generic *E. coli* is performed as well but it is used as a maker for microbiological cleanliness and process monitoring (Canadian Food Inspection Agency, 2015). As constant sampling for STEC is costly, the Canadian government aids producers by providing funds to help offset the costs of the intensive sampling for STEC. Nonetheless, if STEC is positively identified under the CFIA verification sampling plan, then the establishment must perform enhanced sampling at their own expense (Canadian Food Inspection Agency, 2015). It is believed that the intensive sampling of beef for STEC may be responsible for the decrease in the rate of STEC related illnesses as contaminated beef lots are prevented from entering the food chain, but this intensive sampling comes with a significant economic cost (Gill, 2018). The cost of the N60 sampling is justified however, as an outbreak can result in the loss of human lives along with considerable financial losses. But if STEC and the mechanisms allowing for its persistence can be better understood, current interventions could be enhanced or new interventions could be developed to further decrease the risk of STEC contamination and the need for intensive sampling.

1.3 STEC Biofilms:

1.3.1 STEC Biofilms:

Biofilms are communities of microorganisms embedded in a polysaccharide matrix that adhere to a surface or to each other (O'Toole, Kaplan, & Kolter, 2000; Vogeleer, Tremblay, Jubelin, Jacques, & Harel, 2016). The components of the biofilm vary depending on the bacteria producing the biofilm, but generally there is a major polysaccharide component, which in STEC biofilms consists of poly-N-Acetylglucosamine, cellulose, and colanic acid (Vogeleer et al., 2016). Biofilms can also contain other soluble substances excreted from bacteria like exogenous

DNA, quorum sensing molecules, and various proteins (Jefferson, 2004). As biofilms act as a shelter for bacteria, STEC embedded in a biofilm are more resistant to stressors in a beef processing plant. This can result in increased tolerance of STEC to sanitizers, temperature, and desiccation and subsequent contamination of beef products (Nesse et al., 2014; Vogeleer et al., 2016; R. Wang et al., 2012).

1.3.2 Biofilm Formation:

Biofilm formation is a multi-step process where STEC or other biofilm forming bacteria will either initially attach to a surface and form an exopolysaccharide matrix or join an existing biofilm (Dourou et al., 2011; Palmer, Flint, & Brooks, 2007). Initial contact to a surface is the first step in forming a biofilm, which is largely mediated by physical contact with the surface of a contaminated item and a surface in the food processing chain coming into contact. This association can result in reversible attachment followed by irreversible attachment. The reversible attachment of bacteria is the first step in biofilm formation as the bacteria must first contact the surface to form a biofilm, with the likelihood of attachment enhanced by the presence of conditioning films. Which are composed of by a collection of macromolecules like proteins, fats, and carbohydrates which allow the bacteria to more firmly adhere to the surface and increase the likelihood of attachment (Joanna Verran, Packer, Kelly, & Whitehead, 2010; Whitehead & Verran, 2006). In the food industry, bacteria can adhere to stainless steel 304, which is commonly used for the construction of food processing equipment and handling surfaces as it is easily cleaned and resistant to corrosion (Dourou et al., 2011; Joanna Verran et al., 2010). However, the contact of meat products and meat exudate with stainless steel results in the formation of conditioning films composed of biomolecules from the meat proteins and polysaccharides promoting conditions that can enhance bacterial adhesion (Allison, Gilbert,

Lappin-Scott, & Wilson, 2000). Differing composition of conditioning films have been shown to affect the rate of transfer of bacteria to the surface, with some films enhancing and others preventing adhesion (Barnes, Lo, Adams, & Chamberlain, 1999; Palmer et al., 2007). Research has shown that pre-conditioning of stainless-steel surfaces with meat juices, enhances the adhesion of bacteria as a result of a reduction in the surface charge that can impede the contact of bacteria with stainless-steel (Palmer et al., 2007). Other researchers found that saturation of a stainless-steel surface with skim milk proteins prevented the attachment of gram-positive bacteria and reduced the of attachment of *E. coli* (Barnes et al., 1999). The reduction in bacterial attachment is believed to be caused by cross-linking of the skim milk proteins forming a barrier on the food processing surface (Barnes et al., 1999). While conditioning with skim milk proteins prevented the attachment of bacteria, the inclusion of iron in skim milk medium increased adhesion. In this case, iron may act as a bridge cation between the protein and the bacterial surface, thereby promoting attachment (Barnes et al., 1999).

While conditioning films aid in reversible attachment, there are varying theories about how attachment occurs at a molecular level (Stoodley et al., 2002). One theory suggests that Lifshitz-van der Waals forces and electrostatic interactions promote adhesion when bacteria are within 20 nm from the contact surface. At approximately 5 nm the bacteria will begin to adhere to the surface, but during this stage, bacteria can still be washed away (Busscher & Weerkamp, 1987). Irreversible attachment follows, and at this point bacteria can not be easily removed from the surface (Srey et al., 2013). The irreversible attachment is believed to be caused by proteins on the cell surface of the bacteria transferring electrons from their carboxylic functional groups to metals within the adherent surface (Poortinga, Bos, & Busscher, 2001). The rate at which irreversible attachment occurs is strain-dependent and varies among isolates, yet Meinders et al.,

(1995) demonstrated that irreversible attachment can occur in as little as 40-70 seconds (Meinders, Van Der Mei, & Busscher, 1995). Once irreversible attachment has occurred, extra polymeric substances (EPS) are produced and at this point a strong shear force is required to remove the bacteria from the contact surface (Srey et al., 2013). As the EPS develop the biofilm enters the maturation phase, and as it matures to form a mushroom shape or remain as multiple scattered micro-colonies that are separated by channels in which nutrients and waste flow (Figure 2). After formation, the mature biofilm can enter the dispersion phase at which point bacteria can be released into the environment leading to contamination of the environment (Donlan, 2002).

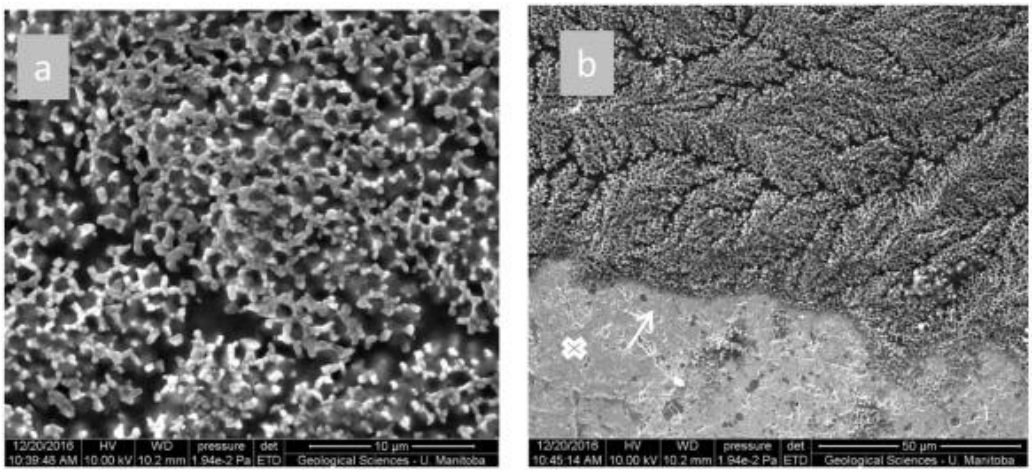


Figure 2. Biofilm formation by E. coli O111-CFS dry biofilm on a stainless-steel coupon A) Biofilm biomass with drainage channels dividing biofilm colonies B) Drainage channels seen within the mass of the biofilm (Adator, Cheng, Holley, McAllister, & Narvaez-Bravo, 2018)

During maturation of the biofilm, many changes in gene expression are observed such as the upregulation of *fimG* and *soxS.* Changes in expression are dependent on environmental factors surrounding the biofilm including differences in temperature, presence of sanitizers, and nutrient availability (Ren, Bedzyk, Thomas, Ye, & Wood, 2004). A loss of cell motility has been observed in both *E. coli* and *Pseudomonas aeruginosa* biofilms, likely because of changes in

flagellar morphology (Ren et al., 2004; Whiteley et al., 2001). With down regulation of genes such as *fimG* and *flhD* can alter flagellar function and morphology leading to losses in cell motility (Pratt & Kolter, 2002; Ren et al., 2004). Depending on the bacterial species, biofilms have been noted to take upwards of 10 days to reach structural maturity (Stoodley et al., 2002). Biofilms formed by *Psuedomonas aeruginosa*, *Pseudomonas flourescens*, *Klebsiella pneumonia* and *Stenotophomonas maltophilia* all required approximately 10 days at 28 ^{o}C in low nutrient medium to reach maturity (Stoodley, Dodds, Boyle, & Lappin-Scott, 1998). Once mature, the biofilm enters the dispersal phase at which point the EPS encapsulating the bacteria is degraded, and cells are shed from the biofilm into the environment (Srey et al., 2013). Biofilm shedding within a food production facility allows for easy dispersal of bacteria into the environment leading to contamination of materials and potential outbreaks. Contamination from biofilms can occur before the dispersion phase (growth < 10 days) and as a result even immature biofilms pose a food safety threat (Dourou et al., 2011; Srey et al., 2013; Vazquez-Sanchez, Galvao, & Oetterer, 2018; R. Wang et al., 2012).

1.3.3 High Event Periods:

As mentioned earlier, biofilms may be involved in High Event Periods (HEP), which are localized events in which STEC are identified in meat processing facilities with a positivity rate statistically greater than 5% ($p > 0.05$) of their established baseline of STEC detection (Food Safety and Inspection Service, 2014). Currently, HEPs are not well understood, as there is still no consensus about what causes an HEP, but current theories include weather, seasonality, animal stress, super-shedder cattle, and biofilms (Arthur, Bono, & Kalchayanand, 2014; Rong et al., 2016; Stanford et al., 2017).

Biofilms are thought to be a possible cause of HEPs as bacterial strains isolated from HEPs show little strain to strain diversity, indicating potential in-house contamination from a single persistent strain (Arthur et al., 2014). Similarly, the increased resistance of STEC within biofilms to sanitizers could enable STEC to be a continuing source of contamination within the beef processing environment (Rong et al., 2016). As bacteria within a biofilm have been observed to spontaneously shed from the biofilm either as a result of stimuli or by reaching the dispersal phase, all of which could contribute to the formation of a HEP (Lim, Song, Park, Lee, & Lee, 2017; Srey et al., 2013).

1.3.4 Biofilm Resistance to Sanitizers:

Sanitizers like quaternary ammonia compounds (QAC), chlorine, and peroxides are authorized for use in beef processing plants to sanitize food contact surfaces (Rong et al., 2016; Vogeleer et al., 2016). Unfortunately, biofilms can increase resistance of bacteria to sanitizers preventing full sterilization of food contact surfaces, a concern for beef processors as trusted sanitization practices may not be effective (Vogeleer et al., 2016). The increased resistance of STEC within biofilms to sanitizers appears to involve both the protective effect of the EPS matrix and changes in gene regulation. The EPS limits the accessibility of the sanitizer to the bacteria in the biofilm due to reduced water activity and penetration of the sanitizer into the biofilm. The EPS also appears to protect bacteria like O157:H7 from osmotic shock due to dissolved solutes within the biofilm (Chen, Lee, & Mao, 2004). While bacteria in a biofilm undergo many changes in gene regulation, some specific changes of direct relevance to sanitizer resistance do take place. These include upregulation of two genes like *hslS*, a heat shock protein which allows for the bacteria to stay viable under normally lethal temperatures (Kitagawa, Matsumura, & Tsuchido, 2000). A second gene, *soxS* also reduces the effectiveness of

superoxides, a form of reactive oxygen species that kills bacteria by oxidation (Vatansever et al., 2013). However, the aforementioned gene regulation changes are not consistent across all *E. coli* strains, as some strains exhibit these traits, and others do not (Ren et al., 2004).

1.3.5 Genetics of Biofilms:

The genetic elements involved in biofilm formation are complex, with further research needed to identify the genetic and regulatory elements influencing the final structure of biofilms. Current research indicates that biofilm formation is strain-dependent, as there seems to be no relationship between serovar and biofilm-forming ability (R. Wang et al., 2012). Additionally, a large proportion of the current literature on biofilm formation focuses on generic *E. coli* strains which are not classified as STEC.

As discussed in section 1.3.2 the life cycle of a biofilm first begins with attachment (reversible then irreversible), microcolony formation, maturation, and then finally dispersion (Stoodley et al., 2002). Research on non-STEC strains of *E. coli* identified 79 genes (1.84% of targets genome) with altered expression in biofilm vs planktonic growth during exponential and stationary growth phases (Schembri, Kjærgaard, & Klemm, 2003). Many of these changes in expression were noted for hypothetical proteins, hydrogenase enzymes, phosphotransferases, outer membrane proteins involved in permeation and adhesion, and metal-binding enzymes (Schembri et al., 2003). Changes in gene expression when transitioning from a planktonic state to a sessile biofilm state alters cell metabolism, as bacteria adapt to stressors (Schembri et al., 2003). The increased expression of outer membrane proteins related to adhesion is likely directly related to bacteria encountering an adherent surface (Schembri et al., 2003). The initial step of reversible adhesion to a substrate is a physical process in which various cellular structures aid in overcoming electrostatic and hydrodynamic forces to allow contact and adhesion of the cell to a

surface. Therefore, it is unlikely that changes in gene expression occur at this point, as the surface has no direct influence on the bacteria.

Once entering the biofilm stage of life it is estimated that over 440 genes alter expression as tested for in two different O157 serovars (Lee, Kim, Cho, Wood, & Lee, 2011). Flagellar synthesis (coded for by *flhDC*) is repressed as the bacteria adhere to a surface, and motility is not required by bacteria within biofilms (Sharma et al., 2016). It is hypothesized that pili encoded for by the *fim* gene play a role in signaling to the bacteria that attachment has occurred, as the expression of pili is induced by adhesion and biofilm formation (Beloin, Roux, & Ghigo, 2008). Pili surface contact has been observed to decrease the production of several different outer membrane proteins, consequently resulting in an increase in the formation of exopolysaccharides, facilitating the formation of matrix that surrounds biofilm microcolonies (Beloin et al., 2008). Curli proteins coded for by the *csg* gene will also be further produced by *E. coli*, enhancing adhesion to the surface and other cells within the biofilm (Sharma et al., 2016). After a microcolony is formed, *E. coli* enters the maturation phase where increased production of EPS and autotransporters occurs. Autotransporters like Antigen 43 encoded for by the *flu* gene are upregulated and facilitate cell to cell communication within the biofilm (Sharma et al., 2016). Genes like *pgaABCD* and *bcsA*, encoding β-1,6-N-acetyl-D-glucosamine (PGA), and cellulose production respectively, are upregulated (Sharma et al., 2016). *OmpC* a gene coding for an outer membrane porin involved in colanic acid formation is also upregulated when *E. coli* forms biofilms (Prigent-Combaret, Vidal, Dorel, & Lejeune, 1999).

Quorum sensing compounds that are used by bacteria for both intra and interspecies communication are also produced, and may increase biofilm mass and initiate the signal that leads to the dispersion of biofilms (Sharma et al., 2016). Quorum sensing compounds are

autoinducers and include N-acyl-homoserine lactones (AI-1) and furanosyl borate diester (AI-2). *E. coli* cannot produce AI-1, but it can sense it as the gene *sdiA* encodes for a homolog of *luxR*, which is the response regulator typically responding to AI-1. Sensing of AI-1 at its critical concentration leads to upregulation of *uvrY* and *csrA* which can promote an increase in biofilm mass (Beloin et al., 2008; Sharma et al., 2016). AI-2 also participates in both inter and intraspecies communication and has been proposed to be responsible for a significant increase in biofilm biomass (Sharma et al., 2016). During maturation, many stress resistance genes are found to be upregulated, such as the *ymgB* which imparts acid resistance upon *E. coli* (Wood, 2009). When gene expression of *E. coli* in biofilms was compared to planktonic cultures, expression of many transport proteins was reduced, possibly accounting for the increased resistance of bacteria cells to antimicrobials within biofilms (Schembri et al., 2003). The *rpoS* gene is also upregulated in biofilms, which is responsible for encoding the S sigma factor protein required for transcription in bacteria (Sharma et al., 2016). The S sigma factor is particularly important in biofilm stress responses as it is needed for the transcription of genes involved in antibiotic, osmotic, ethanol, and peroxide resistance (Hengge-Aronis, Lange, Henneberg, & Fischer, 1993).

The genetic mechanisms involved in biofilm dispersion is still an area of intensive research and of great interest to the food industry. The dispersion of biofilms is classified into passive and active categories. Biofilm passive transfer involves the transfer of bacteria to an external medium due to shear forces (Kaplan, 2010). In contrast, active dispersion involves genetic changes that promote the dispersal of bacteria from the biofilm (Kaplan, 2010). Different types of active dispersion occur, including sloughing, where large portions of the biofilms are sloughed away (Kaplan, 2010). Seeding is a second method of dispersal and involves the

formation of central hollows within a biofilm which are then filled with a high numbers of cells which are released (Kaplan, 2010). The factors that trigger dispersal are still unknown, with theories involving nutrient and environmental cues as well as quorum sensing molecules (Kaplan, 2010; Sharma et al., 2016). Quorum sensing molecules may serve as a signal to initiate dispersion from the biofilm, although the specific AI-1 molecules responsible for dispersion have yet to be identified. In *Pseudomonas aeruginosa* biofilms, N-3-oxo-dodecanoyl homoserine lactone may act as an initiator of sloughing and dispersal, but different bacteria have been noted as using different forms of AI-1 to signal dispersion (Kaplan, 2010). Within differing *E. coli* serovars, mechanisms for detachment are known to vary between EPEC and EAEC serovars. While no genetic mechanisms have been proposed to be responsible for modulation of dispersal, it was found that increased production of dispersin promoted the detachment of EAEC bacteria from intestinal mucosa (Sheikh et al., 2002). This detachment is believed to be caused by the dispersin neutralizing electrostatic forces within the biofilm (Sheikh et al., 2002).

Gene expression within biofilms is still a subject of research with advances in RNA sequencing technology and transcriptome analysis revealing newer information on the signaling mechanism that triggers biofilm formation in *E. coli*. Flow chamber analysis identified changes in the expression of over 600 genes when *E. coli* transitioned from a planktonic state to a biofilm (Schembri et al., 2003). Biofilm formation differs between both STEC and non-STEC strains of *E. coli*. Despite the commonality of genes within these groups, no common expression pattern could be identified to account for differences in biofilm formation (Sauer, 2003). It has recently been identified that small non-coding segments of RNA (sRNA) are responsible for triggering the biofilm phenotype and enabling bacteria to sense changes in response to the environment (i.e., temperature) (Bak et al., 2015). Overexpression of various sRNA related to motility has

been shown to both increase and decrease biofilm formation (Bak et al., 2015). For example, the sRNA DsrA decreased bacterial motility and levels of biofilm formation, whereas the sRNA Och5 decreased bacterial motility, and its expression was linked to a 1.5 fold increase in biofilm formation (Bak et al., 2015). The previously mentioned sRNA segments are but a few of many different sRNA segments shown to influence biofilm formation. It has been observed that most of these sRNA segments strongly interact with the Hfq protein, which serves to both stabilize sRNA and mediate hybridization with targeted mRNA (Bak et al., 2015). The sRNA segments may indirectly influence many other targets as well, as they often interact with other proteins within the bacteria that regulate stress responses such as those encoded by *rpoS* (Bak et al., 2015; Repoila, Majdalani, & Gottesman, 2003). sRNA fragments are additionally believed to be involved in surface adhesion and biofilm formation with sRNA segments like *OmrA* and *OmrB* hinder curli and fimbria production after surface contact (Bak et al., 2015).

1.4 Biofilm as a Source of Cross-Contamination:

Previously, the transfer of bacteria from biofilms has usually focused on the transfer of *Listeria monocytogenes* to ready-to-eat foods (Doijad et al., 2015; Midelet & Carpentier, 2002; Rodriguez & McLandsborough, 2007). But recently, researchers have started to investigate the transfer of STEC in biofilms to beef products. The factors responsible for the transfer of bacterial cells from STEC biofilms to beef have been difficult to identify, but have it has been proposed to depend on strain type, growth rate, moisture content and surface characteristics of the biofilm, and the nature of the tissue in contact with the biofilm (Dourou et al., 2011; Flores et al., 2006; Rodriguez & McLandsborough, 2007).

1.4.1 Temperature and Time Influences of STEC Biofilm Development:

As part of good manufacturing practices, the beef industry maintains a cold chain during production to prevent microbial spoilage and contamination. Temperatures in the cold chain are often maintained at 10 ^{o}C or lower, which restricts the growth of bacteria including O157:H7 serovars (Doyle & Schoeni, 1984). Research performed by Bumunanga et al. (2020) showed that relative biofilm mass on a stainless-steel coupon formed by an O129:H23 STEC serovar increased from ~0.14 to ~0.3 RBB (Relative Biofilm Biomass) over 72 hours at 22 ^{o}C, while a stainless-steel coupon maintained at 10 ^{o}C for 168 hours only had a relative biofilm mass of ~0.09. Other STEC serovars were tested by Bumunang et al. (2020) and all serovars grown on stainless steel coupons showed an increased level of relative biofilm mass at 22 ^{o}C as compared to those grown at 10 ^{o}C. Similar results were obtained by Adator et al. (2018), when measuring biofilm-forming strength using the crystal violet optical density method. The information gathered by Adator et al. (2018) identified that the serovars were able to form a significant biofilm mass over four to six-days at 25 ^{o}C, while they formed minimal biofilm mass at 10 ^{o}C. While lower temperatures reduce the growth rate of O157:H7, research by Dourou et al., 2011 has shown that O157:H7 is still able to adhere to a surface and form biofilms on stainless steel surfaces at temperatures as low as 4 ^{o}C. Dourou et al., 2011 observed that while the numbers of O157:H7 in a biofilm on a stainless-steel coupon continuously increased over 7 days at 4 ^{o}C from 2 Log (CFU/cm^2) to 4 Log (CFU/cm^2) while submerged within a fat-lean homogenate growth medium. At 4 ^{o}C growth of *E. coli* O157:H7 is negligible and the increase in the number of O157:H7 adhered to the coupon could be a result of bacteria contacting the coupon due to the Brownian motion of the surrounding medium and subsequent attachment to the coupon. Alternatively, other meat-associated bacteria in the fat-lean homogenate medium such as

Pseudomonas spp., which is a well-known biofilm forming bacteria, could have entrapped the O157:H7 used to inoculate the media within exopolymers being produced by *Psuedomonas* entrapping and facilitating the attachment of the O157:H7 to the stainless-steel coupon (Dourou et al., 2011). Growth of the STEC in the biofilm was not thought to contribute significantly to the increased microbial count (Dourou et al., 2011). Their research also showed that 2 logs CFU/mL of O157:H7 cells could be transferred at 4 ^{o}C to stainless steel coupons within 30 minutes of contact with ground beef liquid homogenate containing 10^6 CFU/g of O157:H7 (Dourou et al., 2011).

At 15 ^{o}C, O157:H7 attached to stainless steel at levels similar levels to those at 4 ^{o}C, but higher levels of STEC contamination occurred after 7 days. These results were similar to those gathered in work performed by Beauchamp et al. (2012) in which stainless steel coupons were contaminated by compression with pieces of fat inoculated with 10^6 (CFU/cm^2) of *E. coli* O157:H7, or the coupons were inoculated by being submerged in beef fat homogenized (1:10 in sterile water 10^6 CFU/mL) and stored at 4 ^{o}C for 30 minutes. Following contamination of the stainless-steel coupons, biofilm formation by *E. coli* O157:H7 was examined over time. The coupons were then enumerated, and a 10^2 to 10^4 (CFU/cm^2) increase of STEC over a 4 day period was observed, and the STEC population then stabilized at 10^4 (CFU/cm^2) for the remaining 12 days of measurement (Beauchamp et al., 2012). What is apparent across the literature is that there is a temperature effect; as temperature approaches the optimal growth temperature of STEC, biofilm formation is accelerated, while at lower temperatures (like 10 ^{o}C) biofilm formation is reduced. While at lower temperatures formation of a biofilm by STEC is reduced, it is still possible that bacteria in the surrounding media may join the biofilm as a result

of bacterial motility or brownian motion of the media surrounding the biofilm allowing for bacteria to interact with the biofilm.

1.4.2 Effect of Moisture on STEC Survival and Transfer:

Research performed by Møretrø et al. (2010) on STEC survivability at various relative humidity conditions (70, 85 and 90% RH) on stainless steel identified a decrease in STEC survivability at 12 ^{o}C over a 19-day period at 70 and 85% moisture. After seven days post inoculation, STEC were reduced by 6 to 5.5 log CFU on stainless steel kept at 70% humidity and maintained at 12 ^{o}C as compared to those at 98% humidity (Møretrø et al., 2010). At 12 ^{o}C, 70% relative humidity after 19-days of aging, the remaining level of STEC surviving on the coupon decreased to 1 Log CFU. Other findings by Møretrø et al. (2010) also indicated that when coupons were kept at 98% humidity the STEC cultures on the stainless-steel coupons decreased by less than one log after 7 days. The level of STEC may have increased at 98% relative humidity at 12 and 20 ^{o}C, depending on if the bacterial inoculant was resuspended in BHI or water (Møretrø et al., 2010). Flores et al., (2006) found that transfer of STEC bacteria to either beef fat or lean meat occurred more readily if the inoculum was applied to the test surface and dried followed by testing transfer to meat than if the inoculum was applied to the surface allowed to remain moist. The study designed by Flores et al., (2006) was not created to measure the effect of biofilms on transfer to different surfaces, but to show how moisture conditions on a surface impact the transfer of bacteria to beef. In their study an inoculum was either pipetted onto a high-density polypropylene (HDPE) board at 10^5 CFU/mL and then pressed against the meat to measure transfer or the HDPE was dried for 5 minutes in a biological safety cabinet prior to be being pressed against meat. The effect of moisture on the transfer of O157:H7 to beef was found to vary depending on other factors such as whether transfer was occurring from a smooth or

rough surface or if a dry or wet inoculum was used. Overall they found that more bacteria were transferred from a dry surface (Flores et al., 2006). Research investigating the effect of moisture on transfer from dried *L. monocytogenes* biofilms also found higher levels of transfer (10^1) to cheese and bologna than if the biofilm was left moist (Rodriguez & McLandsborough, 2007). The reason for the increased level of transfer from dry biofilms to food products is thought to be caused by decreased adhesive forces between bacteria and the biofilm (Rodriguez & McLandsborough, 2007). The dehydration of the biofilm is theorized to decrease capillary forces within the biofilm, enabling more bacteria to be transferred to meat products (Flemming, 1995). As capillary forces are weakened as the biofilm dries, it is more likely to break apart then if it is maintained in a moist state (Rodríguez et al., 2007).

1.4.3 STEC Transfer to Adipose and Lean Tissue:

Flores et al., (2006) observed that the initial transfer rates of STEC from biofilms on HDPE boards was greater to lean tissue than adipose tissue. Unfortunately, the authors did not offer a reason as to why this difference may have occurred. It is thought that STEC would be more likely to attach to hydrophilic surfaces as the surface of bacteria is negatively charged at a neutral pH. This is due to the negative charges of the phospholipids and dissociation of carboxyl and amino groups which vary with pH and ionic strength of the solution (Li, Shi, Guo, Li, & Xu, 2014; Palmer et al., 2007; Rijnaarts, Norde, Lyklema, & Zehnder, 1999). However, it is believed that if there are more hydrophobic components on the surface of a cell membrane such as flagella, attachment of *E. coli* to hydrophobic surfaces like adipose tissue would be promoted. A study by Friedlander et al. (2015) supported the idea of cellular structures altering hydrophobicity of bacteria by comparing the adherence of wild types of both flagellated and non-flagellated *E. coli* to hydrophobic gold-thiol plated surfaces and to a hydrophilic surface coated

with 11-mercapto-1-undecanol. These models somewhat resemble lean beef tissue which is more hydrophilic and beef fat which is more hydrophobic. Ultimately, a higher level of adherence of flagellated *E. coli* to a more hydrophobic surface was observed, with adhesion to a hydrophilic surface decreased in flagellated *E. coli* as compared to non-flagellated bacteria (Friedlander et al., 2015). Earlier work by Dickson et al., (1989) also showed that *E. coli* O157:H7 adhered more strongly to adipose tissue than lean tissue after dipping either tissue type in an overnight culture grown in Butterfield's phosphate buffer. Their data indicated that O157:H7 adhered more readily to adipose tissue than *Salmonella typhimurium.* It was hypothesized that O157:H7 exhibited greater adherence to adipose tissue due to its lower surface charge than *S. typhimurium,* which was 5 times more negatively charged than O157:H7 (Dickson et al., 1989). The researchers were unable to fully interpret why O157:H7 adhered to adipose tissue more readily than lean tissue, but a similar result was observed in *Serratia marcescens* (Dickson et al., 1989). S_R values were used to quantify relative attachment strength to meat tissues, which are defined by the level of bacteria strongly attached to the meat divided by the sum of the amount of the bacteria strongly and loosely attached (Dickson et al., 1989). The S_R-values for *E. coli* were 0.118 for lean tissue and 0.183 for adipose tissue.

Summary:

STEC biofilms formed within beef processing plants could serve as a source of persistent contamination of beef products, potentially contributing to human illness, death, increased health care costs and monetary losses to the beef industry. Current practices used by the beef industry have been successful in reducing the incidence of STEC cases due to beef contamination, yet the factors that enable STEC to persist in a beef processing environment require further study. It is believed that biofilms which may house STEC, confer tolerance to

thermal treatments, and sanitizers used to reduce the risk of STEC entering the food chain. The increased survivability of STEC within biofilms provides the bacteria with the fortitude to survive and act as a persistent source of contamination. Information as to how conditions like growth time, temperature, moisture affect STEC transfer to different beef tissues (lean and adipose) would provide critical knowledge to improve food safety and reduce the risk of future foodborne STEC outbreaks and illnesses. To answer these questions this work will test the development of STEC biofilms from two different strains grown for differing duration, at various temperatures. The effect biofilm aging has on STEC transfer from biofilms, how differences in moisture affect bacterial survival in biofilm and if differences in growth time and temperature affect transfer from STEC in biofilms to lean and adipose beef tissue.

2 Materials and Methods:

2.1 Bacterial Cultures:

In this study, 13 STEC cultures were tested for their biofilm forming ability, 12 various Beef Processing Facilities (USDA) and 1 from a beef Cattle Feedlot (AAFC) (Table 3). The provided strains were then subsequently tested for biofilm forming strength as summarized in section 2.2. Of these 13, one weak and one strong biofilm forming bacteria were selected for later testing of transfer from biofilms to meat. Both weak and strong biofilm formers were selected to determine an upper limit of bacterial transfer from a strong biofilm former and a lower limit of transfer from a weak biofilm former. All bacteria were stored in the University of Manitoba culture collection and maintained in BHI supplemented with 15% glycerol at -80 ^{o}C .

Table 3. Source and provider of STEC serovars tested.

Serovar	Source	Provider
E. coli O103-2	Beef Processing facility	USDA
E. coli O103-3	Beef Processing facility	USDA
E. coli O45-3	Beef Processing facility	USDA
E. coli O45-4	Beef Processing facility	USDA
E. coli O111-3	Beef Processing facility	USDA
E. coli O111-4	Beef Processing facility	USDA
E. coli O121-3	Beef Processing facility	USDA
E. coli O121-4	Beef Processing facility	USDA
E. coli O145-2	Beef Processing facility	USDA
E. coli O145-3	Beef Processing facility	USDA
E. coli O26-2	Beef Processing facility	USDA
E. coli O26-3	Beef Processing facility	USDA
E. coli O157:H7 R508	Cattle Feedlot	AAFC

USDA: United States Department of Agriculture
AAFC: Agriculture and Agri-Food Canada

2.2 Biofilm Strength Forming Assay:

The biofilm-forming strength of the 12 selected STEC (Table 3) was measured over 2, 4 and 6-day periods at 2, 10, and 25 ^{o}C. The biofilm-forming strength assay was performed using the crystal violet (CV) microplate technique (R. Wang et al., 2012) with minor modifications. Briefly, two experimental replicates were performed with 8 wells of a 96 well microplate being allotted to a given strain. To perform the CV method to assess biofilm formation, cultures were grown overnight at 37 ^{o}C in Lennox Low salt broth (LB:LS, Fisher BioReagents, Pittsburgh PA, USA) to approximately 10^8 CFU/mL. Each culture was then diluted to a concentration of 10^6 CFU/mL in a 96 well flat bottom non-treated polystyrene microplate (FisherBrand, Winnipeg, MB, Canada) containing LB:LS broth. The final volume of culture in the microplate was 200 µL (R. Wang et al., 2012). *E. coli* O157:H7 R508 was used as a positive control, as previous testing by Adator et, al. (2018) identified O157:H7 R5O8 as a strong biofilm former. LB:LS broth containing no culture was used as a negative control. The microplate washing procedure was performed as previously described by Wang et al (R. Wang et al., 2012) with minor modifications. Briefly, after incubation, the culture was removed, and the microplate was washed with 200 µL of phosphate buffered saline (PBS, pH = 7.4) three times and dried for 30 min in a biological safety cabinet. Following drying, the biofilm was fixed using 200 µL of absolute methanol (99.8%, Fisher Chemical, Fair Lawn NJ, USA) for 15 min. Methanol was then removed, and the plate was once again allowed to dry for 15 min in a biological safety cabinet. A 1% aqueous solution of crystal violet (w/v) (90+%, Alfa Aesar, Ontario, Canada) was then added to the microplate well and removed after 15 min. After the crystal violet was removed from the plate it was then washed 3 times with distilled water to remove free crystal violet that was not associated with the biofilm (Adator, Cheng, Holley, McAllister, & Narvaez-Bravo, 2018). After 15-minutes of drying at room temperature, 200µL of glacial acetic acid (99.7%,

Fisher Chemical, Fair Lawn NJ, USA) was added to the microplate well and mixed using a multichannel pipette. The optical density of the solution was then read at 630 nm in a microplate reader (ELx800, Biotek Instruments, Inc., Winooski VT, USA) to determine the biofilm forming strength (Adator et al., 2018). Glacial acetic acid was used to dissolve the biofilm, based on the procedure of (J. Wang et al., 2016)).

Once all data was collected the strains were classified based on their biofilm forming ability (Stepanović, Ćirković, Ranin, & S✓vabić-Vlahović, 2004). Briefly, a blank was used to establish an optical density cut-off point (ODc) which was defined as the pooled mean of all negative control replicates at a given time and temperature plus three standard deviations. Any bacteria which had a mean OD < ODc was then classified as a non-biofilm former. Any serovar with a pooled mean less than (2 x ODc) and greater than the ODc was classified as a weak biofilm former, bacteria with a mean OD > (2 x ODc) and < (ODc x 4) was classified as a moderate biofilm former and finally any bacteria with a mean > (ODc x 4) was classified as a strong biofilm former (Stepanović et al., 2004).

2.3 Experimental Design:

The experimental design of the biofilm transfer study was a full factorial design with nesting. Each experimental unit was prepared in triplicate and overall, two experimental replicates separated in time were performed. The following factors were used to determine the transfer of bacteria to beef: biofilm growth time, temperature, aging time, moisture level, tissue type, and bacterial strain (Table 4).

Another set of coupons was prepared in triplicate, alongside the ones used to test biofilm transfer from the stainless-steel coupon to the meat. These coupons were handled in the same manner and at the same time as the coupons used to assess transfer and were randomly selected

for enumeration. To enumerate the coupons, all conditions outlined in table 4 except for tissue type were used and enumerated as outlined in section 2.5.2

The inoculation of coupons at each growth temperature (2, 10 and 25 ^{o}C) was staggered for each temperature with the 2 ^{o}C set of coupons being inoculated first, the 10 ^{o}C after 8 days and the 25 ^{o}C after 16 days. This staggered approach was adopted to prevent a large overlap of samples on any given day.

Table 4. Full list of factors tested, and *Escherichia coli* strains selected.

Strains	Growth Time (Days)	Growth Temperature (^{o}C)	Aging Time (Days)	Aging Temperature (^{o}C)	Moisture	Tissue
O157:H7 R508	2	2	2	2	Wet	Adipose
O26-2:F2	4	10	4	10	Dry	Lean
	6	25	6	25		
			30			

2.4 Sample preparation:

2.4.2 Coupon Preparation:

Before experimentation stainless steel coupons (SS-304b) of a 2 cm diameter were washed as described by (Ryu et al., 2004). Briefly, coupons were first sonicated at 40 MHz using a Branson 2800 Sonicator (Brookfield, Connecticut) in distilled water for 30 min at 60 ^{o}C, rinsed three times in distilled water and then sonicated in a solution of HC-10 Chlorinated Kleer-Mor alkaline detergent (Ecolab, Mississauga, Ontario, Canada) using the same settings used for the initial rinsing. After sonication in detergent, coupons were rinsed with distilled water and then sonicated in distilled water. Finally, coupons were sonicated under the same conditions in a 15% solution of phosphoric acid (Fisher Chemical, Fair Lawn NJ, USA). Coupons were then rinsed

in distilled water before being placed in an Erlenmeyer flask, covered with aluminum foil, and autoclaved at 121 ^{o}C for 20 min.

2.4.3 Meat Preparation:

Semi tendinous beef cuts (eye round whole approximately 2.5 – 3kg)were purchased at a local grocery market when needed. This beef cut was chosen as it can be purchased with an intact fat cap that can be used as a source of adipose tissue. The meat was prepared by first removing the fat cap and setting the whole lean tissue aside. To remove background flora on the meat, the lean tissue was submerged into a 4 L solution of 5% lactic acid (v/v)(Fisher Chemical, Fair Lawn NJ, USA) for 1 minute. Lactic acid was used to wash the meat, as it is often used to wash carcasses within meat processing environments (Wolf et al., 2012). The outer portion of the beef was then removed using a sterile knife and finally, the lean meat was sectioned into ~2x2x1 cm squares of beef .

The separated fat cap was sprayed with 5% lactic acid and allowed to stand for approximately 15 seconds before being blotted using a non-sterile paper towel. The reduced application time of the lactic acid to the fat was performed to prevent the desiccation of the outer fat surface as it was determined that 15 seconds did not desiccate the fatty tissue as determined by visual inspection during pre-testing experiments (data not presented). As with the lean meat, after treatment, the fat was then cut into ~2x2 cm pieces.

The lean and adipose tissue was sorted into separate vacuum-sealable bags (FlairPak Vacuum Pouch, Flair Flexible Packaging Corporation, Calgary, Canada), and vacuum-packed using a VacMaster VP215 vacuum packager (Overland Park, Kansas). The bags were then sealed under a vacuum and stored at 2 ^{o}C until needed.

2.4.4 Biofilm Growth:

Biofilms were grown using the method described by Adator et al. (2018). Briefly, individual STEC cultures were grown overnight in LB:LS broth and diluted to a concentration of 10^6 CFU/mL. Three stainless steel coupons were then placed in petri-plates (60 x 15 mm) for inoculation with the concavity of the coupon facing downwards. A 10^6 bacterial culture (5 mL) was added to the petri-plate containing the coupons and the plate was placed at one of the specified temperatures (Table 4) (Adator et al., 2018).

2.4.5 Coupon Washing and Maintenance:

After incubation for the specified time (Table 4), coupons were then washed to remove excess broth and loosely adhered bacterial cells. Coupons were washed with 25 mL of Butterfields phosphate buffer by rinsing the coupons three times using a Sartorius Midi Plus pipette (Göttingen, Germany) controller set at the maximum flow setting. The coupon was then placed in a petri-plate with the concavity of the coupon facing downwards. Dry biofilms were generated by leaving the coupons to dry in a biological safety cabinet for 4 h at ambient temperature. The coupon was then aged at the same temperature used for growth for its assigned period. Biofilms were visually monitored for moisture build-up during aging, and wet coupons were kept moist by spraying sterile distilled water on the surface of the coupon. Water was applied using a small spray bottle to deliver 0.23±0.11 mL of water to each coupon.

2.5 Biofilm Transfer:

2.5.1 STEC Transfer from Biofilms to Beef:

A method similar to that used by Flores et al. (2006) was used to determine the number of bacteria transferred to beef from the surface of stainless-steel coupons. The inoculated stainless-steel coupons were arranged in the petri-plate, and a 2x2 cm piece of beef was placed on top of

the coupon. A slip of wax paper was placed over the meat to prevent direct contact with a 50 g weight that was placed on top of the meat to exert 2.69 kPa of pressure. Applying weight to the meat on top of the coupon aided in simulating the weight an intact beef sub-primal would apply to a surface. After, 5 min of contact, the weight and wax paper were removed, and the meat was aseptically transferred into a WhirlPack™ bag. Finally, 9 mL of buffered peptone water (BPW) (Criterion, Santa Maria CA, USA) was transferred into the bag and stomached (Interscience Inc., Markham, ON, Canada) for 1 min before plating. After stomaching the sample was serially diluted (1:10) using 9 mL of BPW and spread plated on MacConkey agar (Criterion, Santa Maria CA, USA) supplemented with 10 mg/L of Novobiocin. The stomached beef samples were then refrigerated at 4 ^{o}C.

2.5.2 Coupon Enumeration:

A set of coupons were used to enumerate STEC using the drop plate technique (Becky, Martin, & Joanna, 2001). Briefly, the inoculated coupons were placed in 60 x15 mm petri-dishes and 9 mL of BPW was added. The coupons in BPW were then sonicated in a Branson 2800 Sonicator for 1 min at 40 MHz. Following sonication, 50 µL of the suspension was plated in 5 separate 10 µL drops onto MacConkey agar. If needed 1:10 serial dilutions were performed by diluting 25 µL of the buffer containing the bacteria into 225 µL of BPW. The samples were then stored at 4 ^{o}C and enriched the following day if no colonies were apparent.

2.5.3 Meat Enrichment:

Meat samples in which STEC could not be detected after plating were enriched to assess STEC survival. Meat pieces were first re-stomached for 1 min and then a 1 mL aliquot of the stomached suspension was transferred into 9 mL of modified Tryptone Soya Broth (mTSB) (Oxoid, Nepean Ontario, Canada). The broth was then incubated at 37 ^{o}C for 48 h and then

streaked onto MacConkey agar supplemented with 10 mg/L of Novobiocin. The plates were then incubated for 24 h at 37 °C, with presumptive colonies being confirmed as outlined below.

Agglutination tests (Rabbit antiserum SSI Diagnostica, Hillerød, Denmark) or PCR was used to verify presumptive STEC colonies as positive. If PCR was needed DNA extraction was performed as described by (Carrillo, Kenwell, Iugovaz, & Oyarzabal, 2017). Wherein, presumptive colonies were selected, placed in 20 µL of lysis buffer (0.25 % SDS, BioRad, Mississauga, Ontario, 0.05M NaOH , Fisher Chemical™ , Ottawa, ON, Canada) at 100 °C for 15 min, and centrifuged at 14,000 g at 4°C for 15 minutes. The supernatant was used as template DNA for PCR using the primers targeting the *wzx* gene of the O-antigen for O26 and O157 serovars listed in Table 5 (DebRoy, Roberts, Valadez, Dudley, & Cutter, 2011). Singelton PCR reactions were then prepared using 125 µL of Qiagen *Taq* PCR Master Mix Kit (Qiagen Canada, Inc., Mississauga, ON, Canada), 0.5 µL of forward and reverser primers and 1.5 µL of template DNA and 10 µL of DNAase free water. The products of the PCR reactions were then resolved using a 1.5% agarose gel (UltraPure™ Agarose, Invitrogen Burlington, ON, Canada).

Table 5. Primer sets used for the identification of serovars.

Serovar	Forward Primer	Reverse Primer
O26	caatgggcggaaattttaga	ataattttctctgccgtcgc
O157	tcgaggtacctgaatctttccttctgt	accagtcttggtgctgctctgaca

2.5.4 STEC Survival on Stainless-Steel Coupons:

Coupons which bacteria were below detectable levels (10^1) by plating were enriched to determine STEC survival. For this purpose, each selected coupon was retained in the original

BPW and incubated at 37 ^{o}C for an additional 48 hours. Afterward, the enriched solution was then streaked on MacConkey agar and the plate was then incubated for 24 hours at 37 ^{o}C.

2.6 Genome Annotation:

Genome sequences for test strains were provided by Zhang et al., 2021 and sequenced using the following protocol. Library prep of the sequence was performed using the NEBNext Ultra II DNA library prep kit by (P. Zhang et al., 2021). Sequencing was performed using an Illumina HiSeq 4000, with quality control of the raw sequencing reads using FASTQC v0.11.8, Trimmomatic v0.39 used to remove adapter sequences and reads with a quality score <20 of <100 bp (base pairs). Genome assembly was performed using SPAdes v3.14.0 using kmer sizes set to 21, 33, 55, 77, 99 and 127 bp. QUAST v5.0.2 was then used to assess the quality of the genome and contigs <500 bp or less than 10x coverage were removed (P. Zhang et al., 2021).

Fasta sequences for both O157:H7 R5O8 and O26-2 were annotated using the RASTtk tool kit available online from the Pathosystems Resource Integration Center (PATRIC). Serovars were chosen from the PATRIC database for annotation of the tested serovars, the taxonomic IDs for the comparison strains for O26 was 991908 and for O157 it was O157:H7 410290. The specialty genes which include genes related to virulence factors, antibiotic resistance, and drug targets, for each sequence was downloaded from PATRIC and the presence or absence of genes known to be involved in biofilm formation was recorded (Table 9).

2.7 Statistical Analysis:

Analysis of the experimental data was conducted using Proc Mixed, and data was transformed using a $Log_{10} + 1$ transformation (SAS Institute Inc., 2020). To compare the transfer of STEC to meat, a factorial model incorporating the main effects of strain, incubation temperature, incubation time, aging time, moisture, and tissue type. All factor interactions were

included in the model with replicates included as a random effect. Least squares mean were generated using the LSMEANS option using restricted maximum likelihood, and least-square means were compared using the Tukey-Kramer method. Denominator degrees of freedom were adjusted using the Satterthwaite procedure. Preliminary analysis revealed that tissue type had no significant effect, nor was it associated with any significant interactions. Therefore, tissue type was removed from the model. Analysis of the coupon enumeration data was performed as stated above, with strain, temperature, incubation time, aging time, and moisture included as main effects in the model and replicate as a random effect. Proportions of samples that could be enumerated or detected for each STEC strain were calculated arithmetically for each of the main effects.

3. Results:

3.1 Biofilm Forming Strength:

At 2 and 10 °C, no serovar formed a detectable biofilm on the polystyrene microplate (Figure 3). Standard deviations for O26-2 reached past the cut-off zone for weak biofilm formation at both 2 and 10 °C, indicating possible weak biofilm formation and adherence, but it did not meet the criteria to be classified as a biofilm former. Additionally, there was no difference between the mean of O26-2 and the weak biofilm forming cutoff ($p > 0.05$).

Strong biofilm formation was only observed at 25 °C with strains O103-3 and O157:H7 R5O8. O157:H7 R5O8 required 4 days to form a strong biofilm while O103-3 required 6 days to form a strong biofilm. Weak biofilm formation was observed in strains: O26-2, O103-2 and O26-3 only at 25 °C after six days of growth in LB:LS. Moderate biofilm formation was identified in, O111-3, O121-3, O145-2 and O145-4. It was found that strains within the same "O" serovar group had different biofilm-forming capacities, O111-3 and O45-4 showed moderate biofilm formation as opposed to their counterparts O111-4 and O45-3 which failed to form a biofilm over time at all temperatures (Figure 3). O26-2 was then chosen for further transfer testing to represent a weak biofilm forming serovar as it maintained consistent formation of a weak biofilm at 25 °C after each growing period (2, 4 and 6 days), while R5O8 was then chosen to represent a strong biofilm forming bacteria as it showed the highest level of biofilm formation and formed a strong biofilm after only 4 days of growth.

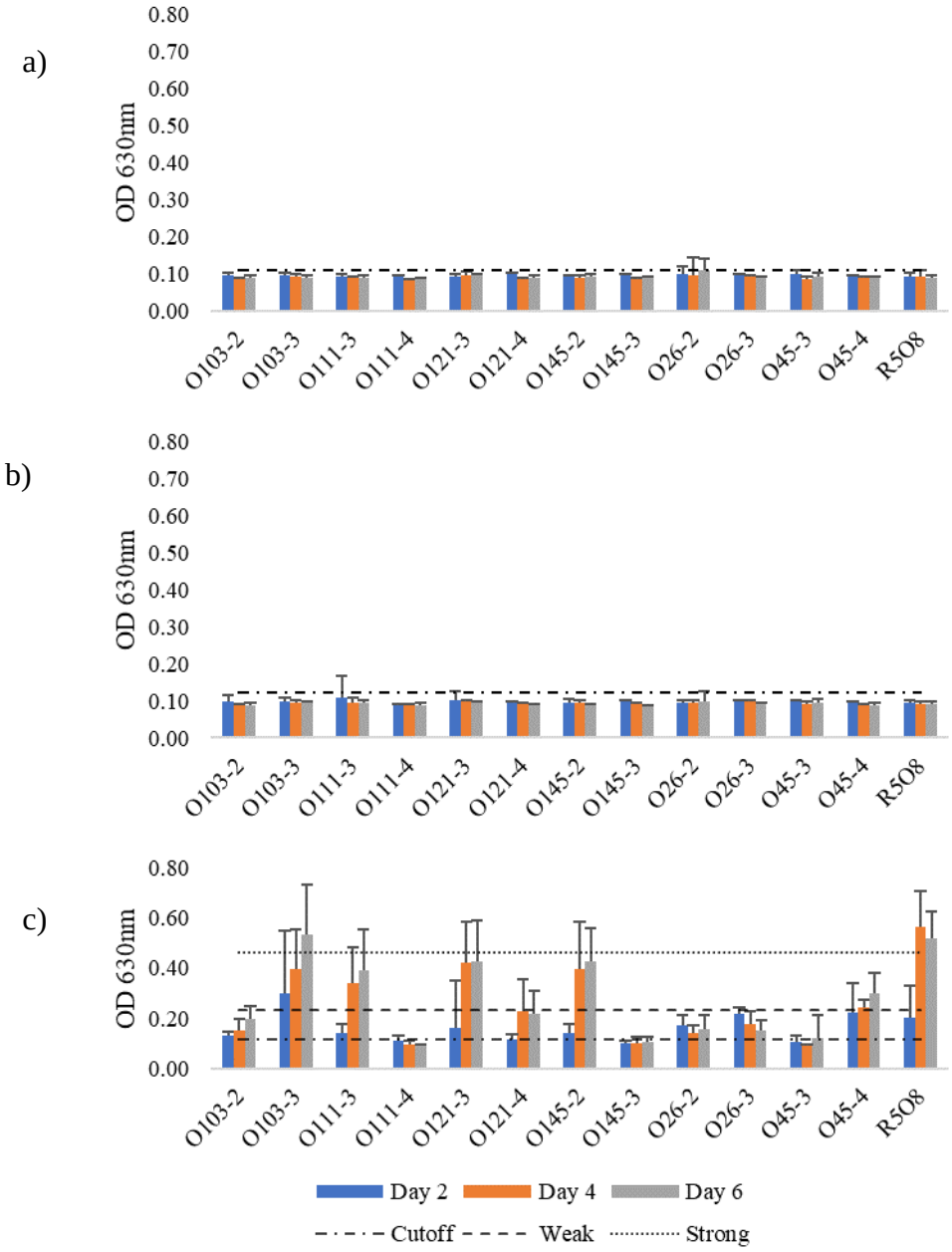


Figure 3. Graphs a, b and c contain the cutoff criteria for the optical density mean at tested temperatures of 2 ºC (a), 10 ºC (b) and 25 ºC (c). Error bars indicate one standard deviation.

3.2 Meat Transfer Data:

A higher level of transfer to beef was observed for O26-2 (1.31 Log CFU/cm^2) a weaker biofilm forming serovar than O157:H7 R5O8 (0.98 Log CFU/cm^2, Table 6, $p < 0.05$). The largest difference in transfer occurred between the two moisture conditions dry (0.33 Log CFU/cm^2) and wet (1.95 Log CFU/cm^2) ($p < 0.01$). At all tested temperatures (2, 10 and 25 ^{o}C) the average level of STEC transfer (both serotypes) from coupons to beef increased ($p < 0.01$) with temperature from 0.53 to 1.90 Log CFU/cm^2 (Table 6). Overall, it was observed that average level of STEC transfer increased ($p < 0.01$) with growth period (2, 4 and 6 days), from 0.86 to 1.42 Log CFU/cm^2. Transfer increased between days 2 to 4 ($p < 0.05$) before plateauing and remaining at a constant level after day 4. Transfer measured from was inversely affected by aging days as transfer decreased as the aging period increased ($p < 0.01$) at each interval (2, 4, 6 and 30 days), initially transfer was 1.52 Log CFU/cm^2 after 2 days and decreased to 0.52 Log CFU/cm^2 after 30 days. Transfer remained stable as aging periods increased (> 1 Log CFU/cm^2) with a notable decrease only after 30 days to 0.52 Log CFU/cm^2 ($p < 0.05$).

Table 6. Least square means of main effects of STEC transferred to beef from stainless-steel coupons.

	Log (CFU/cm^2)
Strain	
O26-2	1.31[b]
O157:H7 R5O8	0.98[a]
SEM	0.09
P-value	<0.01
Temperature, C°	
2	0.53[c]
10	0.98[b]
25	1.90[a]
SEM	0.10
P-value	<0.01
Growing period, d	
2	0.86[b]
4	1.44[a]
6	1.42[ab]
SEM	0.10
P-value	<0.01
Aging days, d	
2	1.52[a]
4	1.29[a]
6	1.24[a]
30	0.52[b]
SEM	0.12
P-value	<0.01
Surface condition	
Wet	1.95[b]
Dry	0.33[a]
SEM	0.09
P-value	< 0.01

[a,b,c] Least squares means within a column lacking a common superscript letter differ ($p < 0.05$).
SEM: Standard error of the mean

Figure 4 shows the significant interaction between STEC biofilm cell transfer to beef at different moisture conditions (dry and wet) and growing periods (2, 4 and 6 days) ($p < 0.05$). The average level of STEC transfer to beef of both STEC serotypes from the wet coupons linearly increased from day 2 (1.42 Log CFU/cm^2) to 6 (2.41 Log CFU/cm^2). Of the wet coupons tested, only transfer between 2 and 4 days differed significantly increasing from 1.42 Log CFU/cm^2 to 2.02 Log CFU/cm^2 ($p < 0.05$) while transfer from the dry coupons remained constant. The dry condition always transferred less than 0.5 Log CFU/cm^2 with transfer not increasing ($p < 0.05$) as growing days continued while transfer from the wet coupons remained above 1.0 Log CFU/cm^2 (Figure 4).

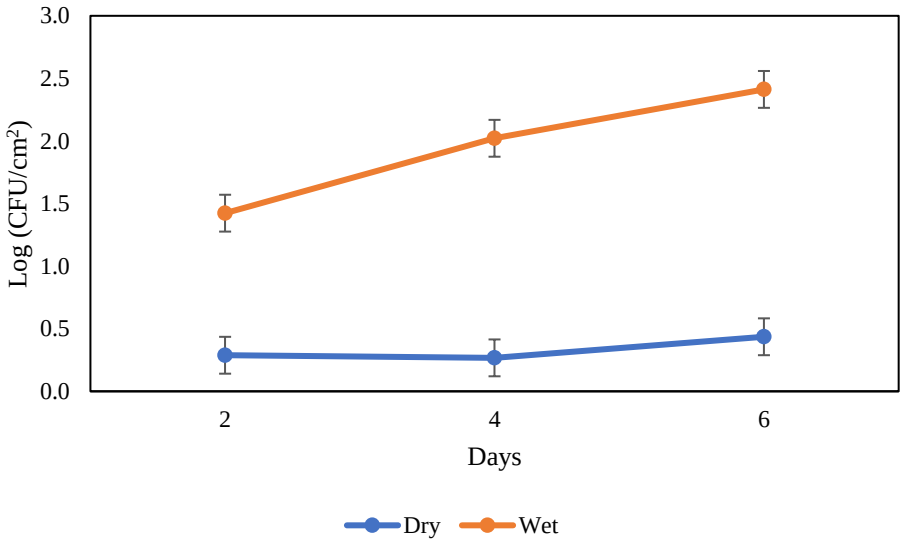
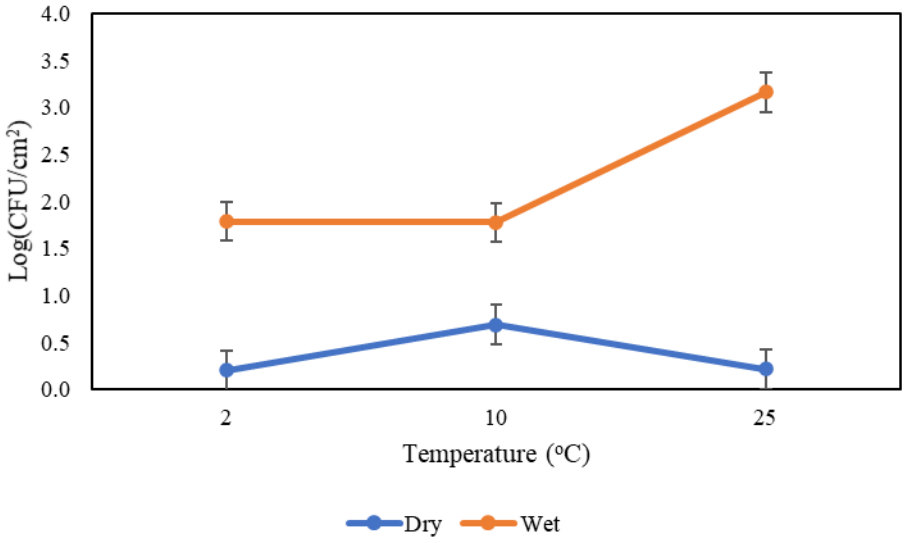


Figure 4. Least squares mean of STEC transferred (Log CFU/cm^2) from wet or dry biofilms formed on stainless-steel coupons to beef after 2, 4 and 6 days of formation. Error bars represent ± one Standard Error.

Figure 5 a & b depicts the 3-factor interaction ($p < 0.05$) between moisture, temperature, and STEC strain (O26-2 (a) and O157:H7 R5O8 (b)). The increase in growth temperature promoted transfer from the moist coupons for both strains ($p < 0.05$). With initial levels of transfer from O157:H7 R5O8 being below 1 Log CFU/cm^2 and increasing to 3.39 Log CFU/cm^2 at 25 ^{o}C. Transfer from O26-2 was higher than O157:H7 R5O8 at lower temperatures (2 and 10 ^{o}C), remaining at ~1.79 Log CFU/cm^2 with transfer from O26-2 increased to 3.16 Log CFU/cm^2 at 25 ^{o}C. A large difference in transfer at 2 ^{o}C between the strains kept wet was observed, with O26-2 transferring 1.79 Log CFU/cm^2 and O157:H7 R5O8 transferring 0.16 Log CFU/cm^2 ($p < 0.05$). Transfer from O157:H7 R5O8 did not change between the wet and dry biofilms at 2 ^{o}C. However, across all the other interactions for the dry coupons shown in Figure 5, the level of transfer did not differ across temperatures.

a)



b)

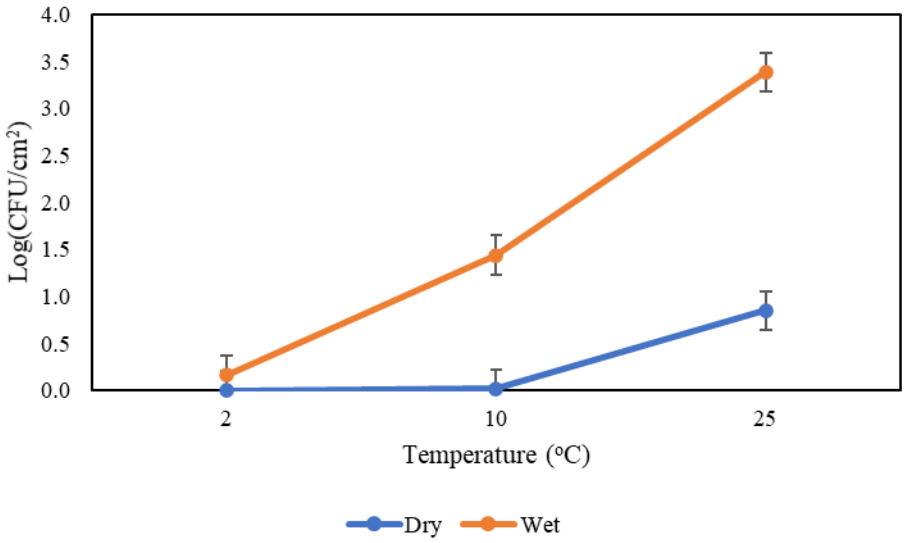
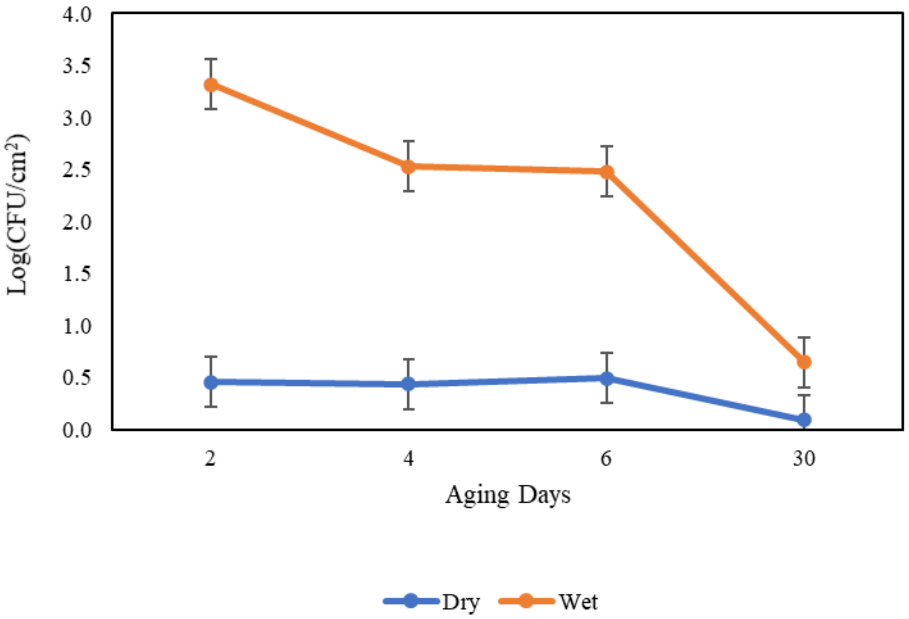


Figure 5. Least squares mean of *E. coli* O26-2 a) and O157:H7 R5O8 b) transferred (Log CFU/cm2) to beef from wet or dry biofilms formed on stainless-steel coupons at 2, 10 and 25 °C. Error bars represent ± one Standard Error.

Figure 6 describes the 3-factor interaction ($p < 0.05$) between aging, moisture, and strain (O26-2 and O157:H7 R5O8). For both strains, transfer was highest from wet coupons after 2 days of aging, with the number of bacteria being transferred to the beef declining during aging ($p < 0.05$). While the transfer from dry coupons for both strains was unaffected as aging continued. On the wet coupons O26-2 transferred 3.32 Log CFU/cm^2 of bacteria after 2-days of aging, while less transfer occurred from O157:H7 R5O8 at 1.97 Log CFU/cm^2 ($p < 0.05$). Transfer from O26-2 decreased to 2.48 Log CFU/cm^2 and remained at ~2.48 Log CFU/cm^2 between days 4 and 6, before decreasing to 0.65 Log CFU/cm^2 after 30 days of aging ($p < 0.05$). Transfer from the wet coupons inoculated with O157:H7 R5O8 to beef remained constant during biofilm aging with a decrease in transfer only observed after 30 days of aging ($p < 0.05$). Coupons kept dry transferred less bacteria to beef than those kept wet ($p < 0.05$). With the exception of 30 days of aging, where O26-2 transferred less ($p < 0.05$) bacteria than O157:H7 R5O8 (0.66 and 1.27 Log CFU/cm^2) respectively. Transfer from the dry coupons remained below 1 Log (CFU/cm^2) for both strains when the coupons were kept dry with no interaction with biofilm aging.

a)



b)

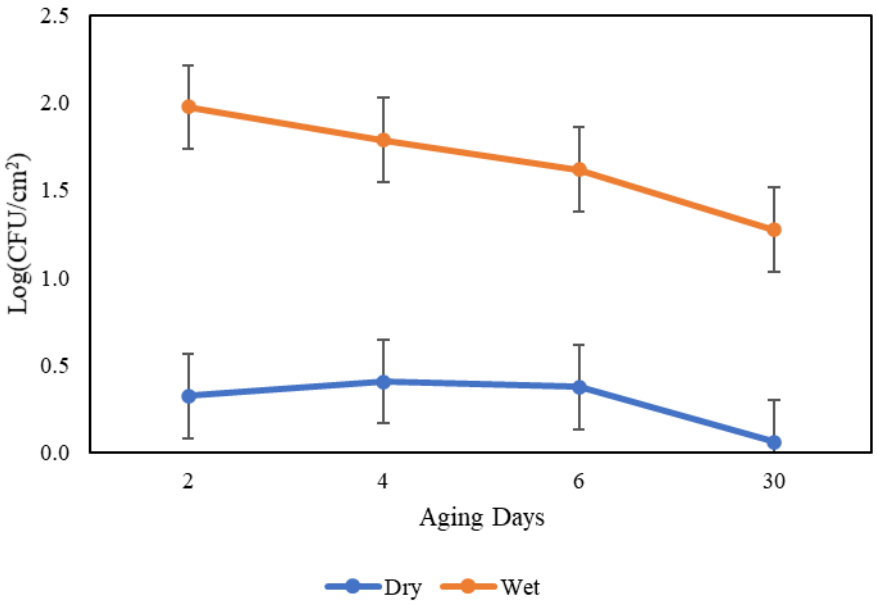


Figure 6. Least squares mean of *E. coli* O26-2 a) and O157:H7 R5O8 b) transferred from wet or dry biofilms aged for 2, 4, 6 and 30 days to on stainless-steel coupons to beef (Log CFU/cm2). Error bars represent ± one Standard Error.

Coupon Enumeration 3.3:

Increases in temperature from 2 to 25 ^{o}C led to an increase of enumerable bacteria from coupons (Table 7, $p < 0.05$), with initial levels of bacteria on the coupon being less than 1 Log CFU/cm^2 at 2 ^{o}C and increasing to 1.29 Log CFU/cm^2 at 25^{o}C. Increasing the period of biofilm growth was also associated with an increase in enumerable bacteria between days 2 (0.36 Log CFU/cm^2) and 6 (0.92 Log CFU/cm^2, $p < 0.05$). As the length of aging increased, the number of bacteria recovered from the coupons decreased with the highest level of bacteria enumerated recorded at 2 days of aging (0.92 Log CFU/cm^2) and then decreasing to 0.41 Log (CFU/cm^2) at day 30 of aging ($p < 0.05$). Wet biofilms also harbored more ($p < 0.05$) bacteria 1.23 Log CFU/cm^2 than dry biofilms (0.04 Log CFU/cm^2).

Table 7. Least square means of main effects for STEC enumerated from stainless steel coupons.

	Log (CFU/cm^2)
Strain	
O26-2	0.65
O157:H7 R5O8	0.62
SEM	0.07
P-value	0.75
Temperature, C°	
2	0.20^{b}
10	0.42^{b}
25	1.29^{a}
SEM	0.09
P-value	<0.01
Growing period, d	
2	0.36^{b}
4	0.67^{ab}
6	0.92^{a}
SEM	0.09
P-value	<0.01
Aging days, d	
2	0.92^{a}
4	0.68^{ab}
6	0.54^{ab}
30	0.41^{b}
SEM	0.10
P-value	<0.01
Surface condition	
Wet	1.23^{a}
Dry	0.04^{b}
SEM	0.07
P-value	< 0.01

^{a,b,c} Least squares means within a column lacking a common superscript letter differ (*p-value* < 0.05).
SEM: Standard error of the mean

As growing temperature increased so did the number of bacteria on the coupons, with strain differences being observed at lower temperatures. At 2 ºC differences in bacterial counts were the largest between tested growing temperatures ($p < 0.05$), initially 0.38 Log CFU/cm^2 of bacteria were recovered from O26-2 biofilms while no bacteria were recovered from the O157:H7 R5O8 biofilms (Figure 7). At 10 ºC the number of bacteria enumerated from the coupons increased to 0.45 Log CFU/cm^2 from O26-2 and to 0.40 Log CFU/cm^2 for O157:H7 R5O8 ($p < 0.05$), resulting in approximately equal levels of bacterial transfer from both strains. At 25ºC the number of bacteria which could be recovered from biofilms on the coupons increased ($p < 0.05$) for both strains (O157:H7 R5O8, 1.46 Log CFU/cm^2 and O26-2, 1.12 Log CFU/cm^2).

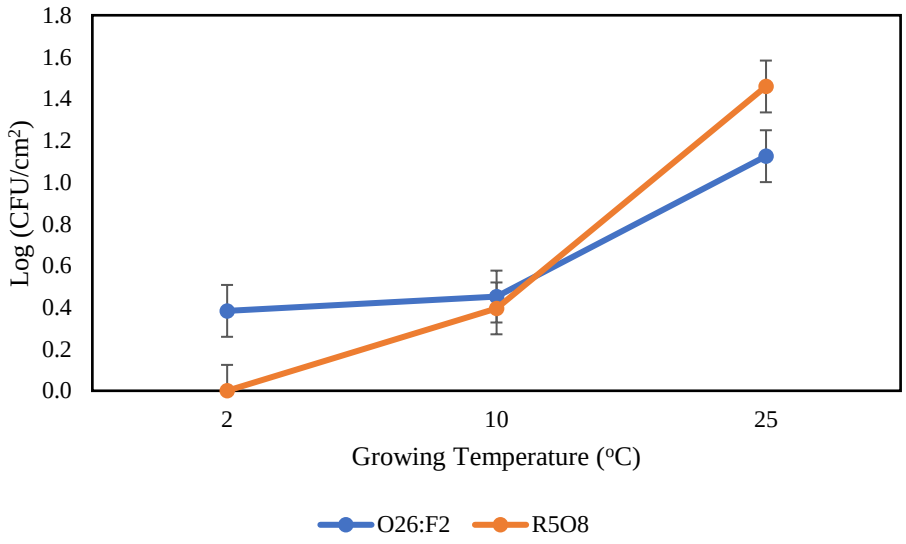


Figure 7. Least squares mean of *E. coli* O26-2 and O157:H7 R5O8 recovered from biofilms grown at 2, 10 and 25 ºC. Error bars represent ± one Standard Error.

Aging day and strain interactions (Figure 8 , $p < 0.05$), show that bacteria enumerated from coupons containing O157:H7 R5O8 held a constant level of bacteria (~0.5 Log CFU/cm^2) as aging days increased with no significant changes at any time, while O26-2 transferred more ($p < 0.05$) bacteria than O157:H7 R5O8 after 2 days of aging (1.2 Log CFU/cm^2). Additionally, the level of bacteria enumerated from coupons inoculated with O26-2 decreased ($p < 0.05$) with aging and was at 0.17 Log CFU/cm^2 after 30 days of aging.

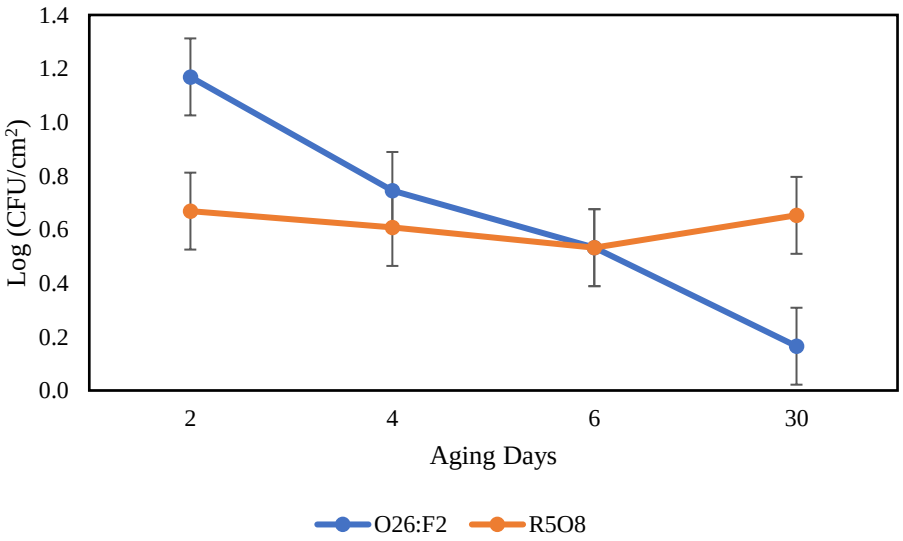


Figure 8. Least squares mean of *E. coli* O26-2 and O157:H7 R5O8 in biofilms on stainless-steel coupons aged for 2, 4, 6 and 30 days. Error bars represent ± one Standard Error.

A significant interaction between moisture level and growing temperature was observed, with levels of bacteria enumerated from the coupons differing between moisture levels at every temperature tested, with the increase in bacterial growth temperature allowing for more bacteria to be recovered from the wet coupons (Figure 9, $p < 0.05$). At 2 ^{o}C wet coupons initially held 0.36 Log CFU/cm^2 while those kept dry held no enumerable bacteria, this difference between the two sets of coupons was not significant. At 10^{o}C, bacteria recovered from the wet coupons increased to 0.84 Log CFU/cm^2 before increasing to 2.49 Log CFU/cm^2 at 25 ^{o}C ($p < 0.05$). While the level of bacteria enumerated from coupons kept wet increased with temperature, dry coupons consistently transferred ~0 Log CFU/cm^2.

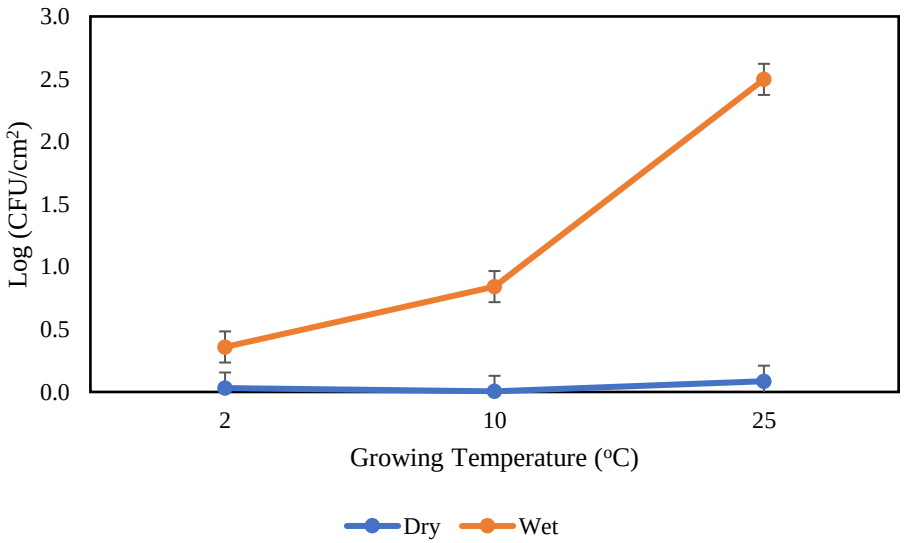


Figure 9. Least squares mean of enumerable bacteria from wet or dry biofilms formed on stainless-steel coupons at 2, 10 and 25 ^{o}C. Error bars represent ± one Standard Error.

The interaction between growing period and moisture shown in Figure 10 and was similar that between growing temperature and moisture condition. The average level of STEC on the coupons that could be recovered increased after each growing day when coupons remained wet, while the drying of the coupons prevented recovery of STEC from the coupons ($p < 0.05$). Recovery of bacteria from wet coupons increased linearly with incubation time, starting at 0.66 Log CFU/cm^2 at day 2 and linearly increased to 1.80 Log CFU/cm^2 after 6 days of growth. The number of bacteria on the dry coupons did not change with respect to aging days, consistently remaining below the limit of detection across all growing periods (<1 Log CFU/cm^2).

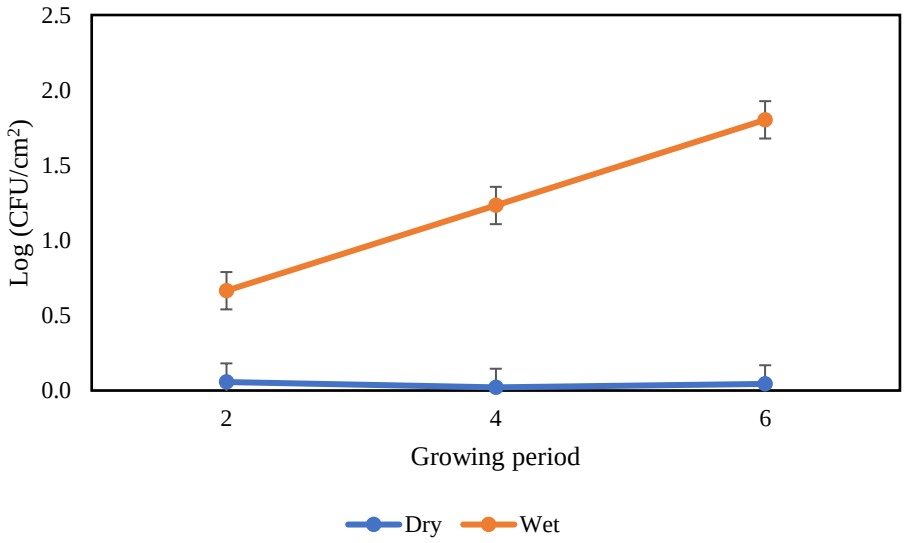


Figure 10. Least squares mean of enumerable bacteria from wet and dry biofilms grown on stainless-steel coupons for 2, 4 and 6 days. Error bars represent ± one Standard Error.

An interaction between aging time and moisture condition was observed as wet biofilms possessed more bacteria than dry biofilms after aging despite bacterial levels on the wet coupons decreasing during aging (Figure 11, $p < 0.05$). Additionally, STEC levels remained below detectable limits (<1 Log CFU/cm^2) on the dry coupons across all aging periods. Initial levels of bacteria recovered from wet biofilms after 2 days of aging was 1.73 Log (CFU/cm^2), with levels subsequently decreasing to 0.82 Log (CFU/cm^2) after 30-days ($p < 0.05$) with aging. Only the decrease in transfer between the wet coupons between 2 and 6 days and 2 and 30 days was significant ($p < 0.05$).

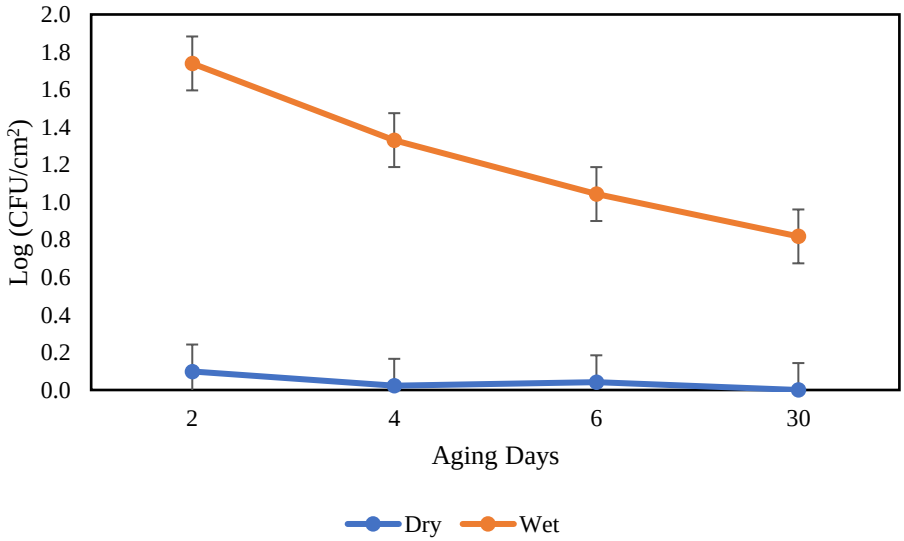


Figure 11. Least squares mean of STEC enumerated from stainless-steel coupons kept dry or wet after aging 2, 4, 6 and 30 days. Error bars represent ± one Standard Error.

3.2 STEC Detection of Negative Samples:

3.2.1 Meat Enrichment Data:

A higher proportion of meat samples possessed enumerable bacteria when the coupon was inoculated with O26-2 (44.8%) than with O157:H7 R5O8 (33.4%, Table 8). Beef pressed against coupons inoculated with O26-2 also had a higher proportion of samples that were positive after enrichment (O26-2 11.6% and O157:H7 R5O8 6.3%). As temperature increased, the transfer of bacteria to beef samples increased. Only 21.0% of samples were contaminated at 2 ^{o}C, whereas over half (54.7%) of the samples had detectable levels of STEC at 25 ^{o}C. As the growth period increased so did the number of beef samples that possessed enumerable bacteria. As the biofilms on coupons were aged, the number of beef samples with enumerable bacteria decreased from 50.1% after 2 days to 16.7% after 30 days. More beef samples possessed enumerable bacteria if they were in contact with wet biofilms as well (62.8%) than with dry biofilms (16.2%). Additionally, when more samples were in contact with wet biofilms (16.8%) were also positive after enrichment as compared to those that contacted dry biofilms (5.1%). There was no apparent difference in the number of enumerable or enriched samples between fat or lean tissue. In total, bacteria were enumerable from 39.1% of beef samples used to test transfer and could be recovered after enrichment from 8.6% of the samples in which STEC could not be detected by enumeration.

Table 8. Proportion of samples with STEC recovery after transfer to beef (without enrichment) and after enrichment.

Main Effect		% (n/N) Recovery without Enrichment [a]	% (n/N) Recovery after 48 hours of Enrichment	Total Number of Samples
Strain	O26-2	44.8 (384/857)	11.6(52/449)	864
	R5O8	33.4(288/863)	6.3(34/544)	
Growing	2	21.0(121/576)	9.2(40/434)	576
Temperature	10	41.6(239/574)	8.6(26/302)	
	25	54.7(312/570)	7.8(20/257)	
Growing Time	2	32.6(187/574)	7.8(29/371)	576
	4	39.4(224/569)	9.1(30/331)	
	6	45.2(261/577)	9.3(27/291)	
Aging Time	2	50.1(215/429)	8.7(18/208)	432
	4	45.1(195/432)	11.6(27/233)	
	6	44.9(190/427)	7.4(17/231)	
	30	16.7(72/432)	7.5(24/321)	
Moisture	Wet	62.8(541/861)	16.8(51/304)	864
	Dry	16.2(139/859)	5.1(35/686)	
Tissue	Fat	40.0(342/858)	10.1(49/486)	864
	Lean	38.2(330/862)	7.3(37/504)	
Total		39.1(672/1720)	8.6(86/993)	1,728

[a] Proportion of samples may not reflect the total number of samples due to missing observations.

3.2.2 Coupon Enrichment Data

Prior to enrichment the proportion of bacteria that were enumerable on the coupons did not differ however after enrichment of the negative coupons (Table 9), 38.0% of samples inoculated with O157:H7 R5O8 samples positive, while only 14.4% of samples inoculated with O26-2 were. Increases in growth temperature, increased the proportion of coupons which held detectable levels of STEC via enumeration, with 8.0% of samples being enumerable at 2 ^{o}C and 39.0% at 25 ^{o}C. The increase in growth temperature resulted in an increase in the number of coupons had recoverable levels of STEC after enrichment increasing from 22.9% to 26.5% between temperatures at 2 and 10 ^{o}C respectively.

Increases in growth time appeared to affect the proportion of coupons which were within the countable range of >1 Log (CFU/cm^2), beginning at 14.7% after 2 days increasing to 30.4% after 6 days. The number of coupons with detectable levels of STEC after enrichment remained at a similar level between days 2 and 4 (22.2%) before increasing to 26.9% after 6. Aging reduced the proportion of coupons that could be enumerated, with the highest level being enumerated after 2 days of aging (33.2%), which decreased to 21.0% after 6 days and to 12.4% after 30 days. Between aging periods of 2 and 6 days the level of coupons which were detectable for STEC remained relatively constant at ~34% recovery, however only 6.3% of the coupons enriched after 30 days of aging held detectable levels of STEC. Moisture had the largest impact on determining whether bacteria could be enumerated from biofilms, with 43.1% of sample positive from wet biofilms, and 2.3% from dry biofilms. Enrichment of the coupons however revealed that over half of the coupons (52.0%) held detectable levels of STEC while STEC was only recovered from 10.5% of the dry coupons. In total, only 22.6% of coupons possessed

enumerable levels of bacteria and of 624 samples which did not have detectable levels of STEC of samples only 25.3% were positive for STEC after enrichment.

Table 9. Proportion of samples with STEC recovery from coupons (without enrichment) and after enrichment

		% (n/N) Recovery without Enrichment	% (n/N) Recovery after 48 hours of Enrichment	Total Number of Treatments
Strain	O26-2	22.6(101/429)	14.4(48/334)	432
	R5O8	22.5(97/431)	38.0(110/290)	
Growing	2	8.0(23/286)	22.9(51/223)	288
Temperature	10	20.5(59/287)	26.5(60/226)	
	25	39.0(112/287)	26.9(47/175)	
Growing Time	2	14.7(42/286)	22.0(54/246)	288
	4	22.6(65/288)	22.4(50/223)	
	6	30.4(87/286)	26.9(54/201)	
Aging Time	2	33.2(71/214)	31.7(40/126)	216
	4	23.6(51/216)	32.0(50/156)	
	6	21.0(45/214)	36.6(56/153)	
	30	12.4(27/217)	6.3(12/189)	
Moisture	Wet	43.1(184/427)	52.0(116/223)	432
	Dry	2.3(10/433)	10.5(42/401)	
Total		22.6(194/860	25.3(158/624)	864

[a] Proportion of samples may not reflect the total number of samples due to missing observations.

3.3 Genome Annotation:

Whole genome sequencing of bacterial isolates aids in their: identification, identifying gene presence, prediction of gene function, comparative analyses and in describing observed phenotypes. The genes listed in Table 10, were selected from the two different sources which studied transcriptomic changes in *E. coli* isolates that transitioned from a planktonic state to a biofilm (Niba, Naka, Nagase, Mori, & Kitakawa, 2007; Schembri et al., 2003). These genes have also been proposed to play a role in biofilm formation by other authors such as Sharma et al., 2016.

Table 10. Specialty genes from literature known to be related to biofilm formation.

Flagellar Synthesis and Components	
flgABCDEFGHIJKLN	[a]
flhABCDE	[a]
fliACDEFGHIJKLMNOPQRST	[a]
Flagellar Motility	
motAB	[a]
Type 1 Fimbriae Synthesis	
fimABCDFGH	[a]
Curli Synthesis	
csgABDEFG	[a]
Lipopolysaccharide Production	
lpcA	[a]
gmhB	[a]
rfaDEFGHP	[a]
Cellular Processes [c]	
btuB	[a]
cheZ	[a]
Crp	[a]
Crr	[a]
cyaA	[a]
degP	[a]
dgkA	[a]
yfgL	[a]
dnaK	[a]
dsbAB	[a]
fruR	[a]

galU	a
gcvA	a
greA	a
hfq	a
hscB	a
hsrA/yieO	a
ihfB	a
Lon	a
mdoH	a
mlrA	a
mltE	a
mog	a
nagA	a
yjhA/nanC	a
nifU	a
nlpD	a
nlpI	a
ompR	a
Pgi	a
proQ	a
ptsI	a
rcsC	a
rpoS	a
sdhC	a
surA	a
tolABR	a
hyaABCDFE	b
hycABF	b
cydAB	b
appAC	b
argCF	b
ilvCM	b
hisCDH	b
treB	b
kgtP	b
stpA	b
crl	b
sdhD	b
artJ	b
pyrBI	b
gltBD	b
gadAB	b
ftn	b

cysCDN	b
ppc	b
glyA	b
oppA	b
folE	b
gatAD	b
serC	b
glnA	b
prsA	b
malK	b
livJ	b
preP	b
nrdEH	b
glgS	b
Uncharacterized [d]	
yhcB	a
yicO	a
ynjC	a
ycfM	a
yciB/ispZ	a
ydeT/fimD	a
yfgA	a
yciM	a
ydaM	a
Open Reading Frames/ Hypothetical Proteins	
yecIH	b
ychH	b
yceP	b
ydcH	b
ylcC	b
yjfNO	b
b2001	b
yccV	b
ygfJ	b
hdeD	b
b2876	b
phnB	b
b1810	b
Heat Shock Proteins	
ibpAB	b
clpB	b
30S Ribosomal Subunit	

rpsCNJSR	[b]
Putative Proteins [e]	
b0836	[b]
b2146	[b]
ydeVW	[b]
yfiA	[b]
adiY	[b]
b1513	[b]
b3001	[b]
xasA	[b]
ytfQR	[b]
b1516	[b]
yhiEMV	[b]
b1973	[b]
b1550	[b]
ykgM	[b]
prpB	[b]
Cold Shock Proteins	
cspA	[b]
Antioxidant Function	
sodA	[b]
50S Ribosomal subunit	
rplBCDMVPYW	[b]
rpmC	[b]
rpmE	[a]
Outer Membrane Proteins	
ompFT	[b]
nmpC	[b]
atpBEG	[b]
flu	[b]

[a] Gene name and annotation from (Niba et al., 2007)

[b] Gene name and annotation from (Schembri et al., 2003)

[c] Genes characterized as relating to cellular processes are those involved in processes of cellular metabolism, DNA replication and reproduction.

[d] Un-characterized genes are identified as being related to biofilm formation, with the function of the gene being predicted.

[e] Putative proteins are those coded for by a gene with sequence similarity to other known genes.

A total of 2249 specialty genes were identified in O157:H7 R508 and 2219 were identified in O26-2, with 1429 specialty genes shared between both strains. Screening of the 189 specialty genes relevant to biofilm formation listed in table 10 identified 78 that were found in both strains with only *tolA* differing, being present in O26-2 and absent in R508.

Table 11. Genes differing between two serovars.

Gene	O26-2	O157:H7 R5O8	Function [a]
tolA	Present	Absent	Component of inner cytoplasmic membrane motor complex. Aiding in bacterial separation through its involvement in cell wall separation. Part of the Tol-Pal system

[a] Gene function identified by (Yakhnina & Bernhardt, 2020)

Table 12. Specialty genes identified in O157:H7 R5O8 and O26-2 relevant to biofilm formation.

R5O8			O26		
adiY	*fliI*	*nlpI*	*adiY*	*fliI*	*nlpI*
artJ	*fliM*	*ompF*	*artJ*	*fliM*	*ompF*
btuB	*fliN*	*ompR*	*btuB*	*fliN*	*ompR*
clpB	*flip*	*ompT*	*clpB*	*flip*	*ompT*
crp	*fliQ*	*oppA*	*crp*	*fliQ*	*oppA*
csgB	*fliR*	*pyrB*	*csgB*	*fliR*	*pyrB*
csgD	*folE*	*rfaG*	*csgD*	*folE*	*rfaG*
csgE	*gadA*	*rfaH*	*csgE*	*gadA*	*rfaH*
csgF	*galU*	*rfaP*	*csgF*	*galU*	*rfaP*
csgG	*glnA*	*rplD*	*csgG*	*glnA*	*rplD*
degP	*glyA*	*rplP*	*degP*	*glyA*	*rplP*
dnaK	*greA*	*rplV*	*dnaK*	*greA*	*rplV*
fimA	*hdeD*	*rpoA*	*fimA*	*hdeD*	*rpoS*
fimB	*hfq*	*rpsC*	*fimB*	*hfq*	*rpsC*
fimC	*hisC*	*rpsJ*	*fimC*	*hisC*	*rpsI*
fimD	*hisD*	*rpsN*	*fimD*	*hisD*	*rpsN*
fimF	*hsrA*	*rpsS*	*fimF*	*hsrA*	*rpsS*
fimG	*hyaA*	*sdhC*	*fimG*	*hyaA*	*sdhC*
fimH	*hyaS*	*sdhD*	*fimH*	*hyaD*	*sdhD*
flh	*ibpB*	*soda*	*flhA*	*ibpB*	*soda*
flhA	*malK*	*surA*	*flhB*	*malK*	*surA*
flhB	*mog*	[a]	*flhC*	*mog*	*tolA*
flhC	*motA*	*tolB*	*flhD*	*motA*	*tolB*
fliC	*motB*	*tolr*	*fliC*	*motB*	*tolR*
fliG	*nanC*	*yhiE*	*fliG*	*nanC*	*yhiE*
fliH	*nlpD*	*ynjC*	*fliH*	*nlpD*	*ynjC*

a Marked space is empty as R5O8 does not contain *tol*A

4 Discussion & Conclusions:

4.1 Biofilm Forming Strength in-vitro assay:

The lack of biofilm formation by the tested strains at lower temperatures using the CV assay (2 and 10 ^{o}C) was not unexpected as testing of various STEC strains by other researchers found that most STEC strains were unable to form biofilms at 10 ^{o}C (Adator et al. 2018). While the STEC strains tested in this research differ from those used by Adator et. al., (2018), a substantive number of STEC strains were tested between both studies, with similar results observed between studies. An inability to form biofilms at lower temperatures (4 and 12 ^{o}C) has also been observed in known psychrotrophic biofilm formers like *Listeria monocytogenes* (Di Bonaventura et al. 2008). The reduction in biofilm formation at lower temperatures can partially be attributed to the effect that sub-optimal growth temperatures have on metabolic processes within the bacteria, as enzymatic reactions are slower and gene expression is altered to enhance bacterial survival at lower temperature through means such as alterations to membrane fluidity (Montville, Matthews, & Kniel, 2012). Not all tested STEC strains in this study may form a strong biofilm on polystyrene as well, and it is possible that more biofilm production may have been observed on other surfaces such as glass or stainless-steel. The effect of surface material on biofilm formation has been assessed using *L. monocytogenes*, where biofilms grown on different surfaces including stainless-steel 304, glass coverslips and polystyrene tissue culture dishes, with biofilm formation at $< 22^{o}$C being highest on glass followed by stainless-steel and polystyrene, respectively (Di Bonaventura et al. 2008).

Lennox broth with low salt (LB:LS) used for the growth and formation of STEC biofilms on polystyrene microplates additionally may have influenced the attachment and formation of biofilms by the tested strains. Different growth mediums have been known to affect biofilm formation of *E. coli*, with media constituents such as the types of amino acids and lipids present

in a media having varied effects on the adhesion of *E. coli* to polystyrene microplates and subsequent biofilm formation (Reisner, Krogfelt, Klein, Zechner, & Molin, 2006). With media such as AB broth, another media similar to M9 media and used in biofilm formation studies (Clark & Maaløe, 1967), supplemented with casamino acids stimulating a higher levels of biofilm formation than LB broth as the increased osmotic conditions of LB broth compared to that of AB broth may hinder biofilm formation (Reisner et al., 2006). Alternatively, certain strain-dependent factors could affect the ability of an individual strains to form a biofilm at lower temperatures including: the production of EPS, presence of curli, other surface proteins and carbohydrates on the bacterial outer membrane (Friedlander et al. 2015, Adator et al. 2018). In this research it was found that at 25 ºC, most of the strains formed a biofilm to differing degrees, with large differences in biofilm formation observed within each O serogroup. Similar results were observed by Wang et al. 2012, in which 10 different STEC strains belonging to O157:H7, O26:H11 and O111:H8 were tested. Within each serogroup, differences in the level of biofilm formation on polystyrene microplates were observed. The results gathered by Wang et al. 2012 and mine suggest that the factors influencing adhesion to polystyrene are not likely tied to the O serotype of STEC. Sequencing of the O157 O-islands by Perna et al. 2001 identified virulence factors related to toxin generation located on O-islands, but not factors related to host adhesion, and proposed that smaller strain-specific islands likely lead to differences in cellular survival and host adhesion (Perna et al. 2001). Growth at 25 ºC, as opposed to 37 ºC has been noted to enhance biofilm formation in strains with small regulatory RNA segments increasing rates of *rpoS* translation inducing transcription of RpoS dependent genes (White-Ziegler et al. 2008). These RNA segments coordinate the expression of genes at room temperature and their activity likely allows for increased biofilm formation at 25 ºC. Additionally, genes like *mlrA* show four

times higher levels of expression at 23 ºC compared to 37 ºC and are directly related to upregulation of curli gene operons (*csgD* and *csgBA)* potentially increasing biofilm formation (White-Ziegler et al., 2008).

4.2 Meat Transfer Data:

All main effects of the meat transfer data (growing temperature, growing days, aging days, and moisture) were found to be significant ($p < 0.05$) except for tissue type (lean or adipose). These results differ from those of Dickson et al. (1989) which identified that *E. coli* O157:H7 more readily adhered to beef adipose tissue than to lean tissue. The lack of observable influences from the two tissue types used in this research (lean and adipose) on bacterial transfer may be the result of two factors. The treatment of ground beef with 2% lactic acid solutions have been shown to cause levels of lipid oxidation that are approximately 4 times higher, 7-days post treatment as compared to a untreated ground beef based on measurements from the TBARS assay (Jimenez-Villarreal, Pohlman, Johnson, & Brown, 2003). Increased levels of lipid oxidation can alter the hydrophobicity of fat as it results in the replacement of double bonds in a fatty acids with hydrophilic hydroxyl groups decreasing the adipose tissues hydrophobicity (Mark, 2012). However, pre-treatment of the meat with lactic acid simulates the real-world preprocessing sanitation meat undergoes and represents a more accurate simulation of the beef surface under typical beef processing conditions. Alternatively, the physiochemical properties of the meat may have been influenced by how it was packaged as it was surrounded by meat purge which is a mixture of water, hemoglobin, and other constituents which the meat would not be surrounded by during processing. The meat purge which surrounds the packaged meat can then increase lipid oxidation rates as the heme present in the hemoglobin can act as a catalyst in the formation of hydroperoxides leading to lipid oxidation (Domínguez et al., 2019).

Strain O26-2 (weak biofilm former) showed a higher level of transfer to beef than O157:H7 R5O8 (strong biofilm former). A potential reason for higher levels of transfer from a weak biofilm forming strain as opposed to a strong one may be due to different level of adherence of the biofilms to the stainless-steel coupons. If the biofilm formed by O26-2 was loosely attached to the stainless-steel coupon, more of biofilm may have been transferred upon contact with a meat surface at once allowing for a higher level of contamination after initial biofilm formation. In contrast to the strong biofilm formed by O157:H7 R5O8 which may adhere to the surface with increased strength preventing a large sudden loss of bacteria at one time, allowing for prolonged survival of the bacteria and more persistent contamination. Overall, it was found that the weak biofilm forming serovar was able to transfer a larger number of bacteria over a short period of time (e.g. after 2 days shown in Figure 5), while the strong biofilm former transferred less bacteria initially. It is possible that transfer from a weak biofilm forming bacteria is like that of a non-biofilm forming bacteria or a non-mature biofilm, but further testing of transfer from bacteria not in biofilms would be needed, additionally the level of EPS formation on the stainless-steel coupon would need to be quantified to determine how much biofilm was present to aid in determining this hypothesis. The tight adhesion of bacteria within biofilms is not unexpected, as biofilms are often difficult to detect during environmental sampling due to their strong attachment to a surface preventing bacteria within them being removed from the surface and then adhering to the swab. Using either a cellulose sponge, cotton or gauze swabs to test for *E. coli* biofilms recovered only 50% of biofilm bacteria (Suwimon et al., 2017). As purposeful swabbing of a surface cannot efficiently detect bacteria contained within a biofilm, it is likely that a non-purposeful action like placing beef on a contaminated surface would not be able to remove all bacteria trapped within a mature strong biofilm. Additionally, adhesion of biofilms to

various surfaces has been known to vary between species and bacterial serovars as well, therefore these results may not be generalizable to all STEC biofilm formers. With biofilm adhesion to silicon rubber differing among strains of *Staphylococcus* (*aureus* and *epidermis*) and *Pseudomonas aeruginosa* as measured by atomic force microscopy (Muszanska et al., 2012). Therefore, a weak biofilm forming bacteria may initially pose a greater risk then a strong biofilm forming bacteria, as greater sudden STEC transfer may occur.

Differences between temperature, growing days, and aging main effects were observed, with transfer increasing as temperature increased from 2 to 25 ^{o}C for both tested strains (Table 6). Biofilm formation also increased with temperature, with the highest level of transfer occurring from biofilms formed at 25 ^{o}C (Table 6). Bumunang et al. (2020) similarly measured increases in biofilm mass and viable bacterial cells within biofilms as their growth period was lengthened at 22 ^{o}C over a 3-day period. This supports the hypothesis that increased time at an optimum temperature for growth, resulted in more bacteria being transferred from biofilms to meat. The decrease in the transfer of bacteria with prolonged aging of the biofilms (Table 6, Figure 6) has been similarly observed in research performed by Adator et al. (2018) where dry biofilm transfer to lettuce was tested. Their research similarly identified that the longer dry biofilms were aged, the lower the transfer of cells from the biofilm to lettuce.

The decrease in culturable bacterial from aged biofilms is likely a result of desiccation. When biofilms are dried, a large portion of the nutrients are no longer accessible due to the lack of water as a solvent to enable nutrient exchange or to facilitate waste removal from adherent bacterial populations. When moisture is present within biofilms it allows bacteria to utilize the EPS matrix as a source of nutrients, promoting survival and propagation of the resident bacteria

(Karygianni et al. 2020). While transfer of STEC from dry biofilms was occasionally observed, large scale transfer of cells was not observed (i.e., > 2 Log CFU/cm^2).

Few dry coupons yielded transferable bacteria to meat while those kept wet did, with 16.2 % of dry samples transferring detectable levels of STEC to beef and only 5.1% of the meat samples contacting dry coupons being positive for STEC after enrichment (Table 8). Initially 62.8% of samples kept wet transferred culturable STEC to beef with an additional 16.8% of samples positive for STEC after enrichment, indicating that STEC was present on the coupons after biofilm growth. STEC may have been present on the dry samples that were negative after enrichment, but they may have been viable but non culturable (VNBC) or too tightly attached to the tissue surface to be detected. These findings contradict those of Rodriguez et al. 2007 who identified that transfer of *L. monocytogenes* cells from biofilms to bologna and cheese was higher from dry biofilms than wet biofilms which transferred ~3 times less bacteria to cheese or bologna. They proposed that dry biofilms had a lower level of internal cellular adhesion resulting in an increased level of transfer from the biofilm (Rodriguez et al. 2007). However, these results cannot be generalized to my study as the genus and species differed, possibly resulting in difference in resistance to desiccation, biofilm structure, growth and as a result the likelihood of cell transfer from biofilms to a food.

Transfer of O157:H7 R5O8 at 2 ^{o}C was considerably lower than transfer of O26-2 at 2 and 10 ^{o}C, (Table 6) but transfer from wet coupons inoculated with O157:H7 R5O8 increased significantly with temperature (Figure 5). The higher transfer of O26-2 at 2 ^{o}C as compared to O157:H7 R5O8 could be due to differences in gene expression related to cold shock responses controlling proteins in the *csp* (cold shock protein) family (Chung et al. 2006), which were identified in both strains and are synthesized in increased amounts at cooler temperatures

(Chung, Bang, & Drake, 2006). Some other cold shock proteins which were not identified may also be responsible for the increased adhesion of O157:H7 R5O8 at 2 ^{o}C. At 10 and 25 ^{o}C, both strains had similar levels of transfer of bacterial cell to meat from wet biofilms.

Strain O157:H7 R5O8 transferred fewer bacteria than O26-2, however transfer from R5O8 was more consistent as the period of aging was prolonged with transfer from the biofilm remaining nearly constant (Figure 6). Alternatively, the difference in biofilm-forming strength, may affect bacterial transfer with aging as more cells were transferred from O157:H7 R5O8 a strong biofilm former than from the weak biofilm forming bacteria as the aging period was prolonged (e.g., greater than 30 days of aging). It is possible that a larger biofilm supported cell viability as more nutrients would be provided via decomposition of the EPS over a longer period of time (Flemming, Neu, & Wozniak, 2007).

4.3 Coupon Enumeration:

I was unable to determine if the number of bacteria on stainless-steel coupons affected the number of bacteria transferred to meat, as levels of bacteria on the coupons after enumeration were lower than the number of bacteria transferred, and it is unclear if this is due to the lack of bacterial recovery from the coupon. However, the overall trends in enumerable bacteria on the coupons largely echoed those of the meat transfer data. Enumeration of bacteria within biofilms on the coupon is likely underestimated as bacteria within biofilm remain tightly adhered to the surface and those cells that are released often remain aggregated and do not separate sufficiently to form single colonies during enumeration, thereby lowering plate counts (Skandamis et al. 2009). The low number of enumerable bacteria on the coupons as compared to the number of bacteria transferred to the beef may be attributed to the meat in the broth acting as a richer medium for bacterial growth and potentially aiding in injured cell recovery and allowing for

increased bacterial growth, thus resulting in increased bacterial recovery during plating. As meat peptone sources in BPW, which would have been introduced into the buffer by stomaching the meat used to test transfer, are known to increase recovery of sub lethally injured *Salmonella Typhimurium* resulting in increased levels of bacterial quantification (Gray et al. 2008). Some researchers have relied on growth kinetics instead of direct enumeration to estimate the number of bacteria within a biofilm, which may have provided better estimates of the bacterial load present on the coupons but such a method is not easily scalable (Wilson et al., 2017). More research is needed to investigate potential causes for enumeration inconsistencies from biofilms.

To gain a clearer picture of how the bacteria in a biofilm on a coupon reflects transfer to a meat more research into resolving the biofilm at different stages of transfer would be valuable. Developments in the recovery of bacteria from the biofilms for quantification could be performed potentially using real-time PCR. Real-time PCR has been used in the quantification of microorganisms in a biofilm in work performed by Zhang et al., (2018) where the bacteria were extracted from a biofilm attached on a tooth (D. Zhang, Shen, Fuente-Nunez, & Haapasalo, 2018). The same method could likely be applicable in this research extracting bacteria from the biofilm adhered to the stainless-steel coupon. Additionally scanning electron microscopy of the formed biofilm and its cross-sections which would help identify which parts of the biofilm are transferred and in confirming the presence of a biofilm on the stainless-steel coupon providing counts related in the abundance of bacteria on the surface (Arnold & Bailey, 2000). The previously mentioned methods would help in inferring bacterial counts and provide estimates of how many bacteria a biofilm can transfer to meat, but it would not be known if the bacteria were alive or dead.

4.4 STEC Survival and Detection:

The proportion of transfer samples that could be enumerated and detected after enrichment was higher for the weak biofilm former, O26-2 (44.8% recovered without enrichment, 11.6% recovered after enrichment) than the strong biofilm former, O157:H7 R508 a (33.4% enumerated, 6.3% enumerated, Table 8). The higher proportion of beef samples able to be enumerated after transfer from O26-2 from a biofilm is likely related to the cells within the weak biofilm having a weaker attachment for the stainless-steel coupon allowing for a larger release of bacteria. These conclusions are supported by direct enumeration of the coupons, as between the two strains (O26-2 and O157:H7 R5O8) similar levels of bacteria were recovered without enrichment (22.6, 22.5% respectively), while the percent recovery of bacteria after enrichment was much higher for O157:H7 R5O8 than O26-2 (O26-2 14.4%, O157:H7 R5O8 38.0%). This supports the conclusion that the strong biofilm former releases fewer cells and require enrichment to be detected.

An alternative explanation for the lower levels of bacteria enumerated from the coupons as compared to what was enumerated from the meat is that the biofilm provides artificially lower counts of bacteria on the coupons due to a tight adherence of the bacteria to the biofilm preventing enumeration of all bacteria adhered to the coupon. Other researchers have similarly identified issues in the enumeration of O157:H7 serovars from coupons due to the tight adherence of bacteria to the biofilm and the surface it is formed on (Skandamis et al., 2009). Methods for enumeration of biofilms such as bead beating generated lower levels of enumerable bacteria from coupons as bacteria were not removed from the coupon or may have flocculated so that they could not be enumerated as separate colonies (Skandamis et al. 2009).

Direct enrichment of the dry coupons identified fewer positive results than expected results from Adator et al. (2018) indicating higher levels of STEC recovery were possible from coupons kept dry compared to the data gathered within my work this experiment. Strains tested by Adator et al. (2018) were able to survive (30-90%) on the stainless-steel coupons for upwards of one month after drying. The high levels of cell recovery from dry coupons after a month in their study as compared to the low recovery (2.3%) of bacteria recovered in the current study may be due to differences in methodologies in the washing step of the coupons. As I used an electronic pipette controller with a much faster flow rate than the pump pipette controller used in the study of Adator et al., (2018). Additionally, a smaller sample size was used by Adator et al., (2018) and only the main effect of strain type was compared in the tabulation of enrichment probabilities; interaction effects also were not considered in the interpretation of the data from both studies involved in determining recovery of bacteria on dry stainless-steel coupons. In my research across both strains nearly 432 dry coupons were gathered for enrichment of dry coupons as opposed to the 144 used by Adator et al., (2018). Additionally, lags upwards of a week in starting the enrichment process in this study may also contribute to lower levels of bacterial recovery after enrichment.

4.5 Genome Annotation:

Genomic annotation of the strains revealed multiple specialty genes related to pathogenicity. Of the genes known to be involved in biofilm formation, only *tolA* was found to differ between the two strains and was present in O26-2 and absent in O157:H7 R5O8. The gene *tolA* is part of a the Tol-Pal system of the peptidoglycan cleaving system and has also been proposed to play a significant role in biofilm formation as it is associated with decreased adhesion, swimming, swarming and EPS production (Ranjith, Ramchiary et al. 2019). Strain

O26-2 was not found to persist for extended periods of time like O157:H7 R508, with recoverable levels of O26-2 decreasing to 0.2 Log (CFU/cm^2) after 30 days of aging. The O157:H7 R5O8 serovar lacked *tolA* which has been noted as being required for biofilm formation; despite the lack of *tolA* O157:H7 R5O8 it was still a strong biofilm former (Ranjith et al. 2019). Overexpression of other genes like *mltB* and *yddW* have been noted as to compensate for the lack of *tolA* allowing for biofilm formation to take place even in the absence of a functioning Tol-Pal system (Yakhnina & Bernhardt, 2020). Alternatively, the annotation performed may not have identified *tolA* in the O157:H7 R5O8 serovar, while other components of the Tol-Pal system were also identified which could also make up for the lack of a functioning *tolA* system. While *tol*A was not identified in the specialty genes output, it may be present in the genome and was not included in the specialty genes output. Additionally, resequencing of O157:H7 R5O8 with increased coverage may help identify the genes presence. A multitude of genes were identified by annotation of both tested strains, but the function of many genes has not been adequately characterized as uncharacterized hypothetical proteins may also have contributed to the ability of O157:H7 R508 to form strong biofilms.

4.6 STEC Biofilm Formation and HEPs:

A high event period (HEP) occurs when an establishment experiences a high number or rate of positive results for *E. coli* O157:H7 in Canada (Canadian Food Inspection Agency, 2015) or Shiga-toxin producing *E. coli* in the U.S.A (Food Safety and Inspection Service, 2014). Biofilms have been noted as a potential cause of HEPs due to the increased level of survivability a biofilm imparts on bacteria and the similarity between bacteria involved in a HEP (R. Wang et al., 2014). In this research sustained transfer from the strong biofilm forming O157:H7 R5O8 was observed over a 30-day period, suggesting that prolonged recurrent contamination from

STEC could persist within a beef processing facility from both wet and, to a lesser extent dry biofilms. Additionally, within the weak biofilm forming O26-2 a high level of transfer was observed initially from the wet biofilm which then decreased sharply as time continued. The sudden increase then decrease in contamination maybe a contamination pattern similar to what is seen in a HEP, however it is unlikely that a weak biofilm forming serovar would produce such a systemic HEP which occurs when STEC contamination continues over a 60-day period (Food Safety and Inspection Service, 2014). A systemic HEP would more likely be caused by a strong biofilm forming bacteria, as contamination from a strong biofilm can persist even after 30-days. It is difficult to conclude if either of the observed contamination patterns from the tested bacteria mimic those of a HEP as the contamination patterns associated with HEPs are not well understood due to the sporadic nature in which they occur within an abattoir (Stanford et al., 2017), and the biofilms formed by both serovars in this research were capable of transferring a large level of bacteria at once. Therefore, biofilms formed by both tested strains could be responsible for a HEP, but more testing would need to be completed such as the effects of environmental changes on the biofilms and the effect they have on transfer. Changes in environmental conditions such as heating and cooling have been known to increase fecal shedding of O157 serovars such as extreme heat in winter (Stanford et al., 2017), and it is possible that changes in the environmental conditions surrounding the biofilm could potentially trigger a HEP.

4.7 Conclusions:

My initial hypotheses suggested that transfer of bacterial cells from dry biofilms to meat would be higher due to decreased cohesive forces within the biofilm. This hypothesis was rejected as it was observed that wet biofilms transferred more STEC and that transfer of STEC

from dry biofilms was infrequent. Furthermore, it was suspected also that due to STEC having a hydrophobic surface, it would adhere more readily to adipose tissue than lean tissue. This hypothesis was also rejected as there was no difference in transfer from STEC biofilms to the two tested tissue types (lean and adipose).

STEC persistence within biofilms poses a risk to beef producers with contamination from biofilms still being possible after a biofilm has been dried and allowed to remain desiccated for over 30 days. Moisture was found to play a major role in sustaining STEC within a biofilm, along with strain differences affecting the number of bacteria transferred to beef. During the slaughter process of beef processing any biofilm present is likely to start wet as a large amount of water is used during processing to keep areas clean, with the biofilm transitioning into a dry biofilm between production periods. Where the biofilm can then be re-hydrated during subsequent production periods serving as a source of contamination during processing steps like the fabrication stage where carcasses are split into primal and sub-primal parts. The strong biofilm-former O157:H7 R5O8 serovar transferred fewer bacteria than the weak biofilm former O26-2 did to the meat; transfer from O157:H7 R5O8 from moist biofilms was more constant over a 30-day period unlike transfer from O26-2 which decreased dramatically after 30 days of aging. Additionally, the number of bacteria transferred from the coupons increased as with growing time (6 days) and temperature (25 °C) increasing the level of STEC that could be enumerated from the coupons or transferred to the beef. STEC contamination from a desiccated biofilm was still shown to occur, however controlling moisture available to STEC in a biofilm can aid in significantly reducing the number of bacteria available to contaminate meat products. The information within this thesis shows that the risk of contamination exists for even a monospecies STEC biofilm deprived of nutrients can pose.

Genome annotation additionally did not yield information that could aid in explaining the differences between biofilm forming strength and transfer between the two tested STEC strains based on the current screen of genes present in the two strains tested. However, expanding the search for different genes involved in biofilm formation and performing additional annotations and transfer testing of STEC strains may aid in identifying potential differences that aid in identifying differences in biofilm formation between the two strains.

5.0 Prospects:

Biofilm contamination is a complex issue with the risk of biofilm contamination still requiring further research to be better understood. While the research presented here demonstrates the tenacity of STEC to contaminate beef from biofilms, scanning electron microscopy is required to reveal the true extent of biofilm formation on the coupons and determine if only microcolonies or fully mature biofilms were produced. Biofilm surveillance and research would also benefit greatly from improved/cost effective methods for reliable sampling and enumeration of bacteria embedded within a biofilm.

Further questions remain, such as what may have occurred if more frequent measurements of biofilms had been taken throughout the aging periods (e.g., every 2-days), an approach that may help model the possible contribution of biofilms to HEPs. Additionally, the biofilms used in this research were grown within LB broth inoculated with the strain of interest, an approach that aided biofilm formation in a reliable and repeatable way. However, this is not how biofilms would develop within meat processing plants, as biofilms are often a consequence of contact with a food matrix containing multiple different species of bacteria. Therefore, it may be interesting to replicate the experiment using contact with a STEC contaminated piece of beef

as the initial seed for biofilm formation, as it may more accurately reflect the initial conditions of biofilm formation.

Characteristic genes differentiating weak and strong biofilm forming bacteria could not be identified between the two-biofilm forming serovars, nor was biofilm formation found to be uniform within serogroups of STEC. Further research involving genome wide association studies would aid in identifying the roles different genes play in biofilm formation within STEC along with aiding in the development of better controls for biofilms. Transcriptomic analysis of STEC developing a biofilm could additionally aid in identifying interactions between expressed genetic components and biofilm development further explaining why STEC lacking genes for biofilm formation are still able to form biofilms. As currently there is no known expression pattern for STEC in biofilms (Sauer, 2003).

Additionally, a risk assessment could be generated from the information gathered in this study which could eventually contribute to the development of a quantitative microbial risk assessment (QMRA) of STEC biofilms for use by beef processors. A QMRA requires experimental data to be gathered to generate a statistical model capable of predicting the risk of microbiological contamination (Health Canada, 2019). However, limited work has focused on creating a QMRA that considers the risk of transfer of STEC from biofilms during beef processing. The development of a QMRA for STEC transfer during beef processing is important, not just as a part of developing sanitation Good Management Practices (GMP) but it could also provide insight into the possible threat biofilms pose and aid in preventing STEC outbreaks.

My research revealed that regardless of strain, desiccation reduced transfer of STEC to beef. While desiccation could not entirely prevent STEC contamination, it does show the effect that prolonged stress may have on the transfer and viability of bacteria within biofilms. Based on

this outcome it shows that keeping work surfaces dry could be a future control to aid in biofilm prevention and subsequently reducing the risk of STEC biofilm contamination, however this may not be feasible during all parts of the slaughter process.

6.0 References:

Adator, E., Cheng, M., Holley, R., McAllister, T., & Narvaez-Bravo, C. (2018). Ability of shiga toxigenic *Escherichia coli* to survive with dry surface biofilms and transfer to fresh lettuce. *International Journal of Food Microbiology*, 52-59.

Allison, D. G., Gilbert, P., Lappin-Scott, H., & Wilson, M. (2000). *Community Structure and Cooperation in Biofilms* (Vol. 7). Cambridge: Cambridge University Press.

American Meat Institute (Producer). (2009, 2020, 02, 01). The Role of an N-60 Sampling Program in Ground Beef Safety. Retrieved from https://www.meatinstitute.org/index.php?ht=a/GetDocumentAction/i/55845

Arnold, J. W., & Bailey, G. W. (2000). Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning electron and atomic force microscopy study. *Poultry Science, 79*(12), 1839-1845. doi:https://doi.org/10.1093/ps/79.12.1839

Arthur, T. M., Bono, J. L., & Kalchayanand, N. (2014). Characterization of *Escherichia coli* O157: H7 Strains from Contaminated Raw Beef Trim during High Event Periods. *Applied and Environmental Microbiology*. doi:10.1128/AEM.03192-13

Bak, G., Lee, J., Suk, S., Kim, D., Young Lee, J., Kim, K.-S., . . . Lee, Y. (2015). Identification of novel sRNAs involved in biofilm formation, motility and fimbriae formation in *Escherichia coli*. *Scientific Reports, 5*(1), 15287. doi:10.1038/srep15287

Barnes, L. M., Lo, M. F., Adams, M. R., & Chamberlain, A. H. L. (1999). Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. *Applied and Environmental Microbiology, 65*(10), 4543--4548. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC91605

Beauchamp, C. S., Dourou, D., Geornaras, I., Yoon, Y., Scanga, J. A., Belk, K. E., . . . Sofos, J. N. (2012). Transfer, Attachment, and Formation of Biofilms by *Escherichia coli* O157:H7 on Meat-Contact Surface Materials. *Journal of Food Science, 77*(6), M343--M347. doi:10.1111/j.1750-3841.2012.02695.x

Becky, H., Martin, H., & Joanna, H. (2001). How to optimize the drop plate method for enumerating bacteria. *Journal of Microbiological Methods, 44*(2), 121-129. doi:https://doi.org/10.1016/S0167-7012(00)00241-4

Beef Industry Food Safety Council. (2016). *Guidance Document for Lotting and Sampling of Beef Products for Pathogen Analysis Beef Industry Food Safety Council*. Retrieved from https://www.bifsco.org/CMDocs/BIFSCO2/Best Practices New/Lotting_and_Sampling_of_Beef_Products_for_Pathogen_Analysis_Final_2016.pdf

Belnap, W. D., & O'Donnell, J. J. (1955). Epidemic Gastroenteritis due to *Escherichia coli* 0-111. A Review of the Literature, with the Epidemiology, Bacteriology, and Clinical findings of a Large Outbreak. *The Journal of Pediatrics, 47*(2), 178--193. doi:10.1016/S0022-3476(55)80029-7

Beloin, C., Roux, A., & Ghigo, J. M. (2008). *Escherichia coli* Biofilms. *Current topics in microbiology and immunology, 322*, 249-289. doi:10.1007/978-3-540-75418-3_12

Bertoldi, B., Richardson, S., Schneider, R. G., Kurdmongkoltham, P., & Schneider, K. R. (2017). *Preventing Foodborne Illness: E. coli "The Big Six"*. Retrieved from IFAS Extension: FSHN13-09

Busscher, H. J., & Weerkamp, A. H. (1987). Specific and non-specific interactions in bacterial adhesion to solid substrata. *FEMS Microbiology Letters, 46*(2), 165--173. doi:10.1111/j.1574-6968.1987.tb02457.x

Canadian Food Inspection Agency. (2015). Annex O: Policy on the Control of *E. coli* O157:H7/NM Contamination in Raw Beef Products. Retrieved from http://www.inspection.gc.ca/food/archived-food-guidance/meat-and-poultry-products/manual-of-procedures/chapter-4/annex-o/eng/1370616273137/1370616333827

Canadian Food Inspection Agency. (2019a). Beef Processing and Inspection. Retrieved from https://inspection.canada.ca/food-safety-for-consumers/canada-s-food-safety-system/beef-processing-and-inspection/eng/1374555766340/1374821164166

Canadian Food Inspection Agency. (2019b). Food recall warnings and allergy alerts. Retrieved from https://inspection.canada.ca/food-recall-warnings-and-allergy-alerts/eng/1351519587174/1351519588221?ay=2018&fr=33&fc=0&fd=0&ft=2

Canadian Food Inspection Agency. (2019c). Sampling procedures. Retrieved from https://www.inspection.gc.ca/preventive-controls/sampling-procedures/eng/1518033335104/1528203403149

Carrillo, C. D., Kenwell, R., Iugovaz, I., & Oyarzabal, O. A. (2017). Recovery of *Campylobacter* spp. from Food and Environmental Sources. In J. Butcher & A. Stintzi (Eds.), *Campylobacter jejuni: Methods and Protocols* (pp. 12-18). New York, NY: Springer New York.

Chen, J., Lee, S. M., & Mao, Y. (2004). Protective effect of exopolysaccharide colanic acid of *Escherichia coli* O157:H7 to osmotic and oxidative stress. *International Journal of Food Microbiology, 93*, 281--286. doi:10.1016/j.ijfoodmicro.2003.12.004

Chung, H. J., Bang, W., & Drake, M. A. (2006). Stress Response of *Escherichia coli*. *Comprehensive Reviews in Food Science and Food Safety, 5*(3), 52-64. doi:10.1111/j.1541-4337.2006.00002.x

Clark, D. J., & Maaløe, O. (1967). DNA replication and the division cycle in *Escherichia coli*. *Journal of Molecular Biology*, *23*(1), 99-112. doi:https://doi.org/10.1016/S0022-2836(67)80070-6

Codex Alimentarius Commission. (1999). General Requirements (Food Hygiene). Codex Alimentarius (Supplement to Volume 1B). In *Recommended International Code of Practice General Principle of Food Hygiene* (Vol. CAC/RCP 1-1969). Rome: Food And Agriculture Organization Of The United Nations World Health Organization.

Cranfield, J. (2013). *Does Canadian beef demand respond to food safety recalls and food quality improvements?* . Retrieved from Guelph: https://www.beefresearch.ca/files/pdf/fact-sheets/canadian_beef_demand_response_to_food_safety_quality_cranfield_sept2013.pdf

DebRoy, C., Roberts, E., Valadez, A. M., Dudley, E. G., & Cutter, C. N. (2011). Detection of Shiga toxin-producing *Escherichia coli* O26, O45, O103, O111, O113, O121, O145, and O157 serogroups by multiplex polymerase chain reaction of the wzx gene of the O-antigen gene cluster. *Foodborne Pathogens and Disease, 8*, 651+. Retrieved from https://link.gale.com/apps/doc/A256281249/HRCA?u=univmanitoba&sid=HRCA&xid=59ca6865

Delannoy, S., Beutin, L., & Fach, P. (2013). Discrimination of Enterohemorrhagic *Escherichia coli* (EHEC) from Non-EHEC Strains Based on Detection of Various Combinations of Type III Effector Genes. *Journal of clinical microbiology, 51*(10), 3257. doi:10.1128/JCM.01471-13

Dickson, J. S., Koohmaraie, M., & Hruska, R. L. (1989). *Cell Surface Charge Characteristics and Their Relationship to Bacterial Attachment to Meat Surfaces*. Retrieved from

Doijad, S. P., Barbuddhe, S. B., Garg, S., Poharkar, K. V., Kalorey, D. R., Kurkure, N. V., . . . Chakraborty, T. (2015). Biofilm-Forming Abilities of *Listeria monocytogenes* Serotypes Isolated from Different Sources. *PloS one, 10*(9), e0137046. doi:10.1371/journal.pone.0137046

Domínguez, R., Pateiro, M., Gagaoua, M., Barba, F. J., Zhang, W., & Lorenzo, J. M. (2019). A Comprehensive Review on Lipid Oxidation in Meat and Meat Products. *Antioxidants (Basel), 8*(10). doi:10.3390/antiox8100429

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging infectious diseases, 8*(9), 881--890. doi:10.3201/eid0809.020063

Dourou, D., Beaucham, C. S., Yoon, Y., Geornaras, I., Belk, K., Smith, G., . . . Sofos, J. (2011). Attachment and biofilm formation by *Escherichia coli* O157:H7 at different temperatures on various food-contact surfaces encountered in beef processing. *International Journal of Food Microbiology*, 262--268.

Doyle, M. P., & Schoeni, J. L. (1984). Survival and Growth Characteristics of *Escherichia coli* Associated with Hemorrhagic Colitis. *Applied and Environmental Microbiology*, 855-856. doi:10.1128/aem.48.4.855-856.1984

Duckworth, B. (2013, March 1, 2013). New owners upgrade safety measures. Retrieved from https://www.producer.com/2013/03/new-owners-upgrade-safety-measures/

Flemming, H.-C. (1995). Sorption Sites In Biofilms. *Water Science and Technology, 32*(8), 27-33. doi:10.1016/0273-1223(96)00004-2

Flemming, H.-C., Neu, T. R., & Wozniak, D. J. (2007). The EPS matrix: the "house of biofilm cells". *Journal of bacteriology, 189*(22), 7945-7947. doi:10.1128/JB.00858-07

Flores, R. A., Tamplin, M. L., Marmer, B. S., Phillips, J. G., & Cooke, P. H. (2006). Transfer Coefficient Models for *Escherichia coli* O157:H7 on Contacts between Beef Tissue and High-Density Polyethylene Surfaces. *69*(6), 1248-1255. doi:10.4315/0362-028x-69.6.1248

Food Safety and Inspection Service. (2012). *Risk Profile for Pathogenic Non-O157 Shiga Toxin-Producing Escherichia coli (non-O157 STEC).* Retrieved from https://www.fsis.usda.gov/sites/default/files/media_file/2020-07/Non_O157_STEC_Risk_Profile_May2012.pdf

Food Safety and Inspection Service. (2014). *FSIS Compliance Guideline for Establishments Sampling Beef Trimmings for Shiga Toxin-Producing Escherichia coli (STEC) Organisms or Virulence Markers*. Retrieved from https://www.fsis.usda.gov/sites/default/files/import/Compliance-Guide-Est-Sampling-STEC.pdf

Food Safety and Inspection Service. (2017). *FSIS Compliance Guideline for Minimizing the Risk of Shiga Toxin-Producing Escherichia coli (STEC) in Raw Beef (including Veal) Processing Operations.* Retrieved from https://www.fsis.usda.gov/wps/wcm/connect/c1217185-1841-4a29-9e7f-8da6dc26d92c/Compliance-Guideline-STEC-Beef-Processing.pdf?MOD=AJPERES

Friedlander, R. S., Vogel, N., & Aizenberg, J. (2015). Role of Flagella in Adhesion of *Escherichia coli* to Abiotic Surfaces. *Langmuir, 31*(22), 6137--6144.

Fu, S., Bai, X., Fan, R., Sun, H., Xu, Y., & Xiong, Y. (2018). Genetic diversity of the enterohaemolysin gene (ehxA) in non-O157 Shiga toxin-producing *Escherichia coli* strains in China. *Scientific Reports, 8*(1), 4233. doi:10.1038/s41598-018-22699-7

Gill, A. (2018). *The decline in Shiga toxin producing Escherichia coli illness in Canada: evidence for a role in changes in beef industry practices*. Retrieved from https://www.cmsa-ascv.ca/documents/Gill_CMSA%20News%20May%202018.pdf

Goldwater, P. N., & Bettelheim, K. A. (2012). Treatment of enterohemorrhagic *Escherichia coli* (EHEC) infection and hemolytic uremic syndrome (HUS). *BMC Medicine, 10*(1), 12. doi:10.1186/1741-7015-10-12

Health Canada. (2019). *Guidance on the Use of Quantitative Microbial Risk Assessment in Drinking Water*. Retrieved from https://www.canada.ca/en/health-canada/services/environmental-workplace-health/reports-publications/water-quality/guidance-qmra-drinking-water.html

Hengge-Aronis, R., Lange, R., Henneberg, N., & Fischer, D. (1993). Osmotic regulation of rpoS-dependent genes in *Escherichia coli*. *Journal of bacteriology, 175*(1), 259. doi:10.1128/jb.175.1.259-265.1993

Jefferson, K. K. (2004). What drives bacteria to produce a biofilm? *FEMS Microbiology Letters, 236*(2), 163-173. doi:10.1111/j.1574-6968.2004.tb09643.x

Jerse, A. E., Yu, J., Tall, B. D., & Kaper, J. B. (1990). A Genetic Locus of Enteropathogenic *Escherichia coli* Necessary for the Production of Attaching and Effacing Lesions on Tissue Culture Cells. *Proceedings of the National Academy of Sciences of the United States of America, 87*(20), 7839--7843. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC54845

Jimenez-Villarreal, J. R., Pohlman, F. W., Johnson, Z. B., & Brown, A. H. (2003). Effects of chlorine dioxide, cetylpyridinium chloride, lactic acid and trisodium phosphate on physical, chemical and sensory properties of ground beef. *Meat Science, 65*(3), 1055-1062. doi:https://doi.org/10.1016/S0309-1740(02)00320-0

Johnson, R. P., Clarke, R. C., Wilson, J. B., Read, S. C., Rahn, K., Renwick, S. A., . . . Gyles, C. L. (1996). *Growing Concerns and Recent Outbreaks Involving Non-0157:H7 Serotypes of Verotoxigenic Escherichia coli*. Retrieved from https://jfoodprotection.org/doi/pdf/10.4315/0362-028X-59.10.1112

Kaplan, J. B. (2010). Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *Journal of dental research, 89*(3), 205-218. doi:10.1177/0022034509359403

Karmali, M. A., Mascarenhas, M., Shen, S., Ziebell, K., Johnson, S., Reid-Smith, R., . . . Kaper, J. B. (2003). Association of Genomic O Island 122 of *Escherichia coli* EDL 933 with Verocytotoxin-Producing *Escherichia coli* Seropathotypes That Are Linked to Epidemic and/or Serious Disease. *Journal Of Clinical Microbiology, 41*(11), 4930--4940. doi:10.1128/JCM.41.11.4930-4940.2003

Kim, J., Nietfeldt, J., & Benson, A. K. (1999). Octamer-based genome scanning distinguishes a unique subpopulation of *Escherichia coli* O157:H7 strains in cattle. *Proceedings of the National Academy of Sciences of the United States of America, 96*(23), 13288--13293. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC23940

Kitagawa, M., Matsumura, Y., & Tsuchido, T. (2000). Small heat shock proteins, IbpA and IbpB, are involved in resistances to heat and superoxide stresses in *Escherichia coli*. *FEMS Microbiology Letters, 184*(2), 165-171. doi:10.1111/j.1574-6968.2000.tb09009.x

Law, D. (2000). The history and evolution of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. *World Journal of Microbiology and Biotechnology, 16*(8), 701--709. doi:10.1023/A:1008927820535

Lee, J.-H., Kim, Y.-G., Cho, M. H., Wood, T. K., & Lee, J. (2011). Transcriptomic analysis for genetic mechanisms of the factors related to biofilm formation in *Escherichia coli* O157:H7. *Current microbiology, 62*(4), 1321-1330. doi:10.1007/s00284-010-9862-4

Lewis, R. J., Corriveau, A., & Usborne, W. R. (2013). [Independent Review of XL Foods Inc. Beef Recall 2012].

Li, L., Shi, X., Guo, X., Li, H., & Xu, C. (2014). Ionic protein-lipid interaction at the plasma membrane: what can the charge do? *Trends in biochemical sciences, 39*(3), 130--140. doi:10.1016/j.tibs.2014.01.002

Lim, J. H., Song, S.-H., Park, H.-S., Lee, J. R., & Lee, S.-M. (2017). Spontaneous detachment of *Streptococcus mutans* biofilm by synergistic effect between zwitterion and sugar alcohol. *Scientific Reports, 7*(1), 8107. doi:10.1038/s41598-017-08558-x

Louise, C. B., & Obrig, T. G. (1995). Specific interaction of *Escherichia coli* O157:H7-derived Shiga-like Toxin ii with Human Renal Endothelial Cells. *Journal of Infectious Diseases, 172*(5), 1397--1401. doi:10.1093/infdis/172.5.1397

Maple Leaf Foods (Producer). (2021, 2021, 07, 03). Foodborne Illness. Retrieved from https://www.mapleleaffoods.com/food-safety/foodborne-illnesses/

Mark, R. P. (2012). Lipids: Functional Properties. In Y. H. Hui (Ed.), *Food Chemistry: Principles and Applications* (3rd ed., pp. 7-11). West Sacremento: Science Technology System.

Mayer, C. L., Leibowitz, C. S., Kurosawa, S., & Stearns-Kurosawa, D. J. (2012). Shiga toxins and the pathophysiology of hemolytic uremic syndrome in humans and animals. *Toxins, 4*(11), 1261-1287. doi:10.3390/toxins4111261

Meinders, J. M., Van Der Mei, H. C., & Busscher, H. J. (1995). Deposition Efficiency and Reversibility of Bacterial Adhesion under Flow. *Journal Of Colloid And Interface Science, 176*, 329--341. doi:https://doi.org/10.1006/jcis.1995.9960

Messens, W., Bolton, D., Frankel, G., Liebana, E., McLauchlin, J., Morabito, S., . . . Threlfall, E. J. (2015). Defining pathogenic verocytotoxin-producing *Escherichia coli* (VTEC) from cases of human infection in the European Union, 2007–2010. *Epidemiology and Infection, 143*(8), 1652-1661. doi:10.1017/S095026881400137X

Midelet, G., & Carpentier, B. (2002). Transfer of Microorganisms, Including *Listeria monocytogenes*, from Various Materials to Beef. *Applied and Environmental Microbiology, 68*(8), 4015--4024.

Montville, T., Matthews, K., & Kniel, K. (2012). Microbial Growth, Survival, and Death in Foods. In A. S. Microbiolgy (Ed.), *Food Microbiology: An Introduction* (pp. 31-33). Washington: American Society Microbiolgy.

Møretrø, T., Heir, E., Mo, K. R., Habimana, O., Abdelgani, A., & Langsrud, S. (2010). Factors affecting survival of Shigatoxin-producing *Escherichia coli* on abiotic surfaces. *International Journal of Food Microbiology, 138*(1), 71-77. doi:https://doi.org/10.1016/j.ijfoodmicro.2010.01.002

Moxley, R. A., & Smith, D. R. (2010). Attaching-effacing *Escherichia coli* Infections in Cattle. *Veterinary Clinics: Food Animal Practices, 26*, 29-56. doi:10.1016/j.cvfa.2009.10.011

Muszanska, A. K., Nejadnik, M. R., Chen, Y., van den Heuvel, E. R., Busscher, H. J., van der Mei, H. C., & Norde, W. (2012). Bacterial Adhesion Forces with Substratum Surfaces and the Susceptibility of Biofilms to Antibiotics. *Antimicrob Agents Chemother, 56*(9), 4961-4964. doi:10.1128/aac.00431-12

Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev, 11*(1), 142-201. doi:10.1128/cmr.11.1.142

National Enteric Surveillance Program. (2019). *Annual Summary 2019*. Public Health Agency of Canada

Nesse, L. L., Sekse, C., Berg, K., Johannesen, K. C. S., Solheim, H., Vestby, L. K., & Urdahl, A. M. (2014). Potentially pathogenic *Escherichia coli* can form a biofilm under conditions relevant to the food production chain. *Applied and Environmental Microbiology, 80*(7), 2042--2049. doi:10.1128/AEM.03331-13

Niba, E. T. E., Naka, Y., Nagase, M., Mori, H., & Kitakawa, M. (2007). A genome-wide approach to identify the genes involved in biofilm formation in *E. coli*. *DNA research : an international journal for rapid publication of reports on genes and genomes, 14*(6), 237-246. doi:10.1093/dnares/dsm024

O'Brien, A., Lively Thomas, A., & Chen Melinda, E. (1983). *Escherichia coli* O157:H7 Strains Associtated with Haemorragic Colitis in the United States Produce a *Shigella Dysenteriae* 1 (Shiga) Like Cytotoxin. *The Lancet, 321*(8326), 702-702. Retrieved from https://doi.org/10.1016/S0140-6736(83)91987-6

O'Toole, G., Kaplan, H. B., & Kolter, R. (2000). Biofilm Formation as Microbial Development. *Annual Review of Microbiology, 54*(1), 49--79. doi:10.1146/annurev.micro.54.1.49

Ogura, Y., Gotoh, Y., Itoh, T., Sato, M. P., Seto, K., Yoshino, S., . . . Hayashi, T. (2017). Population structure of *Escherichia coli O26:H11* with recent and repeated stx2 acquisition in multiple lineages. *Microbial Genomics, 3*(11). doi:https://doi.org/10.1099/mgen.0.000141

Ölmez, H., & Temur, S. D. (2010). Effects of different sanitizing treatments on biofilms and attachment of Escherichia coli and *Listeria monocytogenes* on green leaf lettuce. *LWT - Food Science and Technology, 43*(6), 964-970. doi:https://doi.org/10.1016/j.lwt.2010.02.005

Palmer, J., Flint, S., & Brooks, J. (2007). Bacterial cell attachment, the beginning of a biofilm. *Journal of Industrial Microbiology and Biotechnology, 34*(9), 577--588. doi:10.1007/s10295-007-0234-4

Pintara, A. P., Guglielmino, C. J. D., Rathnayake, I. U., Huygens, F., Jennison, A. V., & Patel, R. (2018). Molecular Prediction of the O157:H-Negative Phenotype Prevalent in Australian Shiga Toxin-Producing Escherichia coli Cases Improves Concordance of *In Silico* Serotyping with Phenotypic Motility. *Journal Of Clinical Microbiology, 56*(4), e01906-01917. doi:doi:10.1128/JCM.01906-17

Poortinga, A. T., Bos, R., & Busscher, H. J. (2001). Charge Transfer during *Staphylococcal* Adhesion to TiNOX Coatings with Different Specific Resistivity. *Biophysical Chemistry, 91*(3), 273--279. doi:10.1016/S0301-4622(01)00177-6

Pratt, L., & Kolter, R. (2002). Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Journal of Molecular Microbiology, 30*(2), 285--293.

Prigent-Combaret, C., Vidal, O., Dorel, C., & Lejeune, P. (1999). Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *Journal of bacteriology, 181*(19), 5993-6002. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC103626/

Reisner, A., Krogfelt, K. A., Klein, B. M., Zechner, E. L., & Molin, S. (2006). In Vitro Biofilm Formation of Commensal and Pathogenic *Escherichia coli* Strains: Impact of Environmental and Genetic Factors. *Journal of bacteriology, 188*(10), 3572-3581. doi:10.1128/jb.188.10.3572-3581.2006

Ren, D., Bedzyk, L. A., Thomas, S. M., Ye, R. W., & Wood, T. K. (2004). Gene expression in *Escherichia coli* biofilms. *Applied Microbiology and Biotechnology, 64*(4), 515--524.

Repoila, F., Majdalani, N., & Gottesman, S. (2003). Small non-coding RNAs, co-ordinators of adaptation processes in *Escherichia coli*: the RpoS paradigm. *Molecular Microbiology, 48*(4), 855-861. doi:10.1046/j.1365-2958.2003.03454.x

Rijnaarts, H. H. M., Norde, W., Lyklema, J., & Zehnder, A. J. B. (1999). DLVO and steric contributions to bacterial deposition in media of different ionic strengths. *Colloids and Surfaces B: Biointerfaces, 14*(1-4), 179--195. doi:10.1016/S0927-7765(99)00035-1

Rodríguez, A., Autio, W. R., & Mclandsborough, L. A. (2007). Effect of Biofilm Dryness on the Transfer of *Listeria monocytogenes* Biofilms Grown on Stainless Steel to Bologna and Hard Salami. *Journal of Food Protection, 70*(11), 2480-2484. doi:10.4315/0362-028x-70.11.2480

Rodriguez, A., & McLandsborough, L. A. (2007). Evaluation of the transfer of *Listeria monocytogenes* from surfaces to foods. *Journal of Food Protection, 70*, 600--606.

Rong, W., E., L. B., M., B. J., W., S. J., Norasak, K., & M., A. T. (2016). *Escherichia coli* O157:H7 Strains Isolated from High-Event Period Beef Contamination Have Strong Biofilm-Forming Ability and Low Sanitizer Susceptibility, Which Are Associated with High pO157 Plasmid Copy Number. *79*(11), 1875-1883. doi:10.4315/0362-028x.Jfp-16-113

Ryu, J. H., Kim, H., Frank, J. F., & Beuchat, L. R. (2004). Attachment and biofilm formation on stainless steel by *Escherichia coli* O157:H7 as affected by curli production. *Letters in Applied Microbiology, 39*(4), 359-362. doi:10.1111/j.1472-765X.2004.01591.x

SAS Institute Inc. (Producer). (2020). The MIXED Procedure. Retrieved from https://documentation.sas.com/doc/en/statug/15.2/statug_mixed_syntax01.htm

Sauer, K. (2003). The genomics and proteomics of biofilm formation. *Genome biology, 4*(6), 219-219. doi:10.1186/gb-2003-4-6-219

Schembri, M. A., Kjærgaard, K., & Klemm, P. (2003). Global gene expression in *Escherichia coli* biofilms. *Molecular Microbiology, 48*(1), 253-267. doi:10.1046/j.1365-2958.2003.03432.x

Servin, A. (2014). Pathogenesis of Human Diffusely Adhering *Escherichia coli* Expressing Afa/Dr Adhesins (Afa/Dr DAEC): Current Insights and Future Challenges. *CLinical Microbiology Reviews, 27*, 823-869. doi:10.1128/CMR.00036-14

Sharma, G., Sharma, S., Sharma, P., Chandola, D., Dang, S., Gupta, S., & Gabrani, R. (2016). *Escherichia coli* biofilm: development and therapeutic strategies. *Journal of Applied Microbiology, 121*(2), 309-319. doi:10.1111/jam.13078

Sheikh, J., Czeczulin, J. R., Harrington, S., Hicks, S., Henderson, I. R., Le Bouguénec, C., . . . Nataro, J. P. (2002). A novel dispersin protein in enteroaggregative *Escherichia coli*. *The Journal of clinical investigation, 110*(9), 1329-1337. doi:10.1172/JCI16172

Skandamis, P. N., Stopforth, J. D., Ashton, L. V., Geornaras, I., Kendall, P. A., & Sofos, J. N. (2009). *Escherichia coli* O157:H7 survival, biofilm formation and acid tolerance under simulated slaughter plant moist and dry conditions. *Food Microbiology, 26*(1), 112-119. doi:https://doi.org/10.1016/j.fm.2008.09.001

Smith, D. R. (2014). Vaccination of Cattle against *Escherichia coli* O157:H7. *Microbiol Spectr, 2*(6). doi:10.1128/microbiolspec.EHEC-0006-2013

Srey, S., Jahid, I. K., & Ha, S.-D. (2013). Biofilm formation in food industries: A food safety concern. *Food Control, 31*(2), 572-585. doi:https://doi.org/10.1016/j.foodcont.2012.12.001

Stanford, K., Reuter, T., Bach, S. J., Chui, L., Ma, A., Conrad, C. C., . . . McAllister, T. A. (2017). Effect of severe weather events on the shedding of Shiga toxigenic *Escherichia coli* in slaughter cattle and phenotype of serogroup O157 isolates. *FEMS Microbiology Ecology, 93*(9). doi:10.1093/femsec/fix098

Stepanović, S., Ćirković, I., Ranin, L., & S✓vabić-Vlahović, M. (2004). Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *38*(5), 428-432. doi:10.1111/j.1472-765X.2004.01513.x

Stoodley, P., Dodds, I., Boyle, J. D., & Lappin-Scott, H. M. (1998). Influence of hydrodynamics and nutrients on biofilm structure. *Journal of Applied Microbiology, 85*(S1), 19S-28S. doi:10.1111/j.1365-2672.1998.tb05279.x

Stoodley, P., Sauer, K., Davies, D. G., & Costerton, J. W. (2002). Biofilms as Complex Differentiated Communities. *Annual Review of Microbiology, 56*(1), 187--209. doi:10.1146/annurev.micro.56.012302.160705

Suwimon, K., Thamolwan, L., Orapin, P., Yuphakhun, C., Hajime, T., & Punnida, T. (2017). Effect of swabbing techniques on the efficiency of bacterial recovery from food contact surfaces. *Food Control, 77*, 139-144. doi:https://doi.org/10.1016/j.foodcont.2017.02.013

The Marler Clark Network (Producer). (2008). Jack in the Box *E. coli* Outbreak Lawsuits - Western States (1993). Retrieved from https://marlerclark.com/news_events/jack-in-the-box-e-coli-outbreak-western-states

The Marler Clark Network. (2020). McDonald's Restaurant Hamburger 1982. Retrieved from http://www.outbreakdatabase.com/details/mcdonalds-restaurant-hamburger-1982/

Trabulsi, L. R., Keller, R., & Tardelli Gomes, T. A. (2002). Typical and Atypical Enteropathogenic *Escherichia coli*. *Emerging infectious diseases, 8*(5), 508-513. doi:10.3201/eid0805.010385

United States Department of Agriculture (Producer). (2018, 2019, Dec, 16). Recall Summaries 2018. Retrieved from https://www.fsis.usda.gov/wps/portal/fsis/topics/recalls-and-public-health-alerts/recall-summaries

Vatansever, F., de Melo, W. C. M. A., Avci, P., Vecchio, D., Sadasivam, M., Gupta, A., . . . Hamblin, M. R. (2013). Antimicrobial strategies centered around reactive oxygen species--bactericidal antibiotics, photodynamic therapy, and beyond. *FEMS microbiology reviews, 37*(6), 955-989. doi:10.1111/1574-6976.12026

Vazquez-Sanchez, D., Galvao, J. A., & Oetterer, M. (2018). Contamination sources, biofilm-forming ability and biocide resistance of Shiga toxin-producing *Escherichia coli* O157:H7 and non-O157 isolated from tilapia-processing facilities. *Journal of Food Safety, 38*(3).

Verran, J., Boyd, R. D., Hall, K. E., & West, R. (2010). The Detection of Microorganisms and Organic Material on Stainless Steel Food Contact Surfaces. doi:10.1080/08927010290006736

Verran, J., Packer, A., Kelly, P., & Whitehead, K. A. (2010). Titanium-coating of stainless steel as an aid to improved cleanability. *International Journal of Food Microbiology, 141*, S134-S139. doi:10.1016/J.IJFOODMICRO.2010.04.027

Vogeleer, P., Tremblay, Y. D. N., Jubelin, G., Jacques, M., & Harel, J. (2016). Biofilm-Forming Abilities of Shiga Toxin-Producing *Escherichia coli* Isolates Associated with Human Infections. doi:10.1128/AEM.02983-15

Wang, J., Stanford, K., McAllister, T., Johnson, R., Chen, J., Hou, H., . . . Niu, Y. (2016). Biofilm Formation, Virulence Gene Profiles, and Antimicrobial Resistance of Nine Serogroups of Non-O157 Shiga Toxin-Producing *Escherichia coli*. *Foodborne Pathogens and Disease, 13*. doi:10.1089/fpd.2015.2099

Wang, R., Bono, J., Kalchayanand, N., Shackelford, S., & Harhay, D. (2012). Biofilm Formation by Shiga Toxin-Producing *Escherichia coli* O157:H7 and Non-O157 Strains and Their Tolerance to Sanitizers Commonly Used in the Food Processing Environment. *Journal of Food Protection, 75*(8), 1418-1428. doi:10.4315/0362-028X.JFP-11-427

Wang, R., Kalchayanand, N., King, D. A., Luedtke, B. E., Bosilevac, J. M., & Arthur, T. M. (2014). Biofilm Formation and Sanitizer Resistance of *Escherichia coli* O157:H7 Strains Isolated from "High Event Period" Meat Contamination†. *Journal of Food Protection, 77*(11), 1982-1987. doi:10.4315/0362-028x.Jfp-14-253

Wheeler, T. L., Kalchayanand, N., & Bosilevac, J. M. (2014). Pre- and post-harvest interventions to reduce pathogen contamination in the U.S. beef industry. *Meat Science, 98*(3), 372-382. doi:https://doi.org/10.1016/j.meatsci.2014.06.026

White-Ziegler, C. A., Um, S., Pérez, N. M., Berns, A. L., Malhowski, A. J., & Young, S. (2008). Low temperature (23 degrees C) increases expression of biofilm-, cold-shock- and RpoS-dependent genes in *Escherichia coli* K-12. *Microbiology (Reading), 154*(Pt 1), 148-166. doi:10.1099/mic.0.2007/012021-0

Whitehead, K. A., & Verran, J. (2006). The effect of surface topography on the retention of microorganisms. *Food and Bioproducts Processing*: Elsevier.

Whiteley, M., Bangera, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S., & Greenberg, E. P. (2001). Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature, 413*(6858), 860--864. doi:10.1038/35101627

Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., Sandoval, J., . . . Holmes, A. E. (2017). Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review. *Research & reviews. Journal of engineering and technology, 6*(4). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6133255/

Winfield, M. D., & Groisman, E. A. (2003). Role of Nonhost Environments in the Lifestyles of *Salmonella* and *Escherichia coli*. *Applied and Environmental Microbiology, 69*(7), 3687-3694. doi:doi:10.1128/AEM.69.7.3687-3694.2003

Wolf, M. J., Miller, M. F., Parks, A. R., Loneragan, G. H., Garmyn, A. J., Thompson, L. D., . . . Brashears, M. M. (2012). Validation Comparing the Effectiveness of a Lactic Acid Dip with a Lactic Acid Spray for Reducing *Escherichia coli* O157:H7, *Salmonella*, and Non-O157 Shiga Toxigenic *Escherichia coli* on Beef Trim and Ground Beef. *Journal of Food Protection, 75*(11), 1968-1973. doi:10.4315/0362-028x.Jfp-12-038

Wood, T. K. (2009). Insights on *Escherichia coli* biofilm formation and inhibition from whole-transcriptome profiling. *Environmental microbiology, 11*(1), 1-15. doi:10.1111/j.1462-2920.2008.01768.x

Yakhnina, A. A., & Bernhardt, T. G. (2020). The Tol-Pal system is required for peptidoglycan-cleaving enzymes to complete bacterial cell division. *Proc Natl Acad Sci U S A, 117*(12), 6777-6783. doi:10.1073/pnas.1919267117

Zhang, D., Shen, Y., Fuente-Nunez, C. d. l., & Haapasalo, M. (2018). In vitro evaluation by quantitative real-time PCR and culturing of the effectiveness of disinfection of multispecies biofilms in root canals by two irrigation systems. *Clinical Oral Investigations, 23*, 913-920.

Zhang, P., Essendoubi, S., Keenliside, J., Reuter, T., Stanford, K., King, R., & Yang, X. (2021). Genome Sequences of 104 *Escherichia coli O157:H7* Isolates from Pigs, Cattle, and Pork Production Environments in Alberta, Canada. *Microbiology Resource Announcements*. doi:https://doi.org/10.1128/MRA.01320-20