Shiga Toxigenic *Escherichia coli* (STEC) Multispecies Biofilm Formation and its Persistence Mechanisms in Beef Processing Facilities

By

Yuchen Nan

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Department of Food and Human Nutritional Sciences

University of Manitoba

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Abstract

Introduction: Shiga-toxigenic *Escherichia coli* (STEC) are critical enteric pathogens linked to severe foodborne illnesses outbreaks. A potential mechanism for STEC survival and persistence is the formation of biofilms and association with other bacteria in the beef fabrication environment.

Methods: This research involved two STEC serogroups studies with different biofilm-forming abilities, including strong biofilm producer STEC O103:H2 (99-2076) and a moderate biofilm former STEC O157:H7 (1934). One LAB: T1 (*Carnobacterium piscicola* + *Lactobacillus bulgaricus*), and two SP: T2 (*Comamonas koreensis* + *Raoultella terrigena*); T3 (*Pseudomonas aeruginosa* + *C. koreensis*) were tested for their ability to form multispecies biofilms with STEC. STEC single-species biofilms were included as controls (T4). In both studies, the selected STEC serogroups interactions with LAB or SP multispecies biofilms on TPU and SS coupons were evaluated at 10 °C and 25 °C under wet and dry conditions after 6, 30 & 60 d of storage. The capacity of STEC transfer to beef was assessed, and STEC survival within single and multispecies biofilm was assessed by enrichment.

Results: At 25 °C, multispecies biofilm mixture *P. aeruginosa* + *C. koreensis* (T3) showed antagonistic interactions against STEC, decreasing O103:H2 and O157:H7 cell transfer to beef by 2.54 $\log_{10}$ CFU/g ($P < 0.001$) and 1.76 $\log_{10}$ CFU/g ($P < 0.001$), respectively. At 25 ºC, no interactions ($P > 0.05$) against O103:H2 or/ O157:H7 were found on biofilms combining, *C. piscicola* + *L. bulgaricus* (T1) and *C. koreensis* + *R. terrigena* (T2). At 10 °C, none of the multispecies biofilms altered the cell transfer of O103:H2 or/ O157:H7 to beef ($P > 0.05$) compared to the control positive. A greater extent of STEC viable cell transfer to beef from fresher (6 d), moist biofilm, on the TPU surface was observed. No beef contamination with STEC was detected from 60 d old dry biofilms. After enriching the 60 d dry biofilm, the highest overall STEC survival rate was observed from 10 °C multispecies biofilm *R. terrigena* + *C. koreensis* (dry-T2).

Conclusion: The present study demonstrated that the risk of STEC contaminating beef can be influenced by bacterial species composition, adherent surface, humidity, and the age of biofilms.

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Dedication

To my grandfather Qingxian Nan, who guided me to the field of Food Science and triggered my passion for food microbiology.

Thesis Organization

This thesis is divided into five chapters and includes two manuscripts, which are located in chapters three and four.

The first chapter introduces Shiga-toxigenic *E. coli* (STEC), causing beef contamination through biofilm formation. The research hypothesis and objectives are described at the end of the chapter.

The second chapter is a literature review that includes the background information on STEC-caused foodborne diseases, cattle processing procedures and the environment in the beef industry, and STEC biofilm formation mechanisms. The synergistic and antagonistic effects of spoilage and lactic acid bacteria on STEC containing multispecies biofilm formation and STEC persistence are also included in this chapter.

Chapter three was accepted by the Frontiers Journal, entitled "Formation and transfer of multi-species biofilms containing *E. coli* O103:H2 on food contact surfaces to beef" by Yuchen Nan, Argenis Rodas-Gonzalez, Kim Stanford, Celine Nadon, Xianqin Yang, Tim McAllister, Claudia Narváez-Bravo. The biofilm was developed on two types of food contact surfaces, and stored under different times, temperatures, and humidity to evaluate *E. coli* O103:H2 transfer to beef and persistence.

Chapter four describes the extent to which *E. coli* O157:H7 multispecies biofilm caused beef contamination and persistence from two different food contact surfaces after different storage times, temperatures, and humidity conditions. This chapter was prepared for submission to the Journal of Food Protection, entitled "Influence of lactic acid and spoilage bacteria on *E. coli* O157:H7 biofilms on food contact surfaces and their transfer to beef" by Yuchen Nan, Argenis Rodas-Gonzalez, Kim Stanford, Celine Nadon, Xianqin Yang, Tim McAllister, Claudia Narváez-Bravo.

Chapter five summarizes the main discussion and overall conclusion for the whole thesis.

Moreover, it states the recommendations for future studies.

Contributions of Authors

Contributions in the manuscript at Chapter two:

CNB and TM developed this project. CNB supervised the students and lab work. YN designed the study and performed the experiment. ARG analyzed the data. YN drafted the manuscript with the helps from KS, CN, XY, TM, and CNB. All authors listed have made a significant and direct contribution to this project and approved it for publication.

Contributions in the manuscript at Chapter Three:

CNB and TM developed this project. CNB supervised the students and lab work. YN designed the study and performed the experiment. ARG analyzed the data. YN drafted the manuscript with the helps from KS, CN, XY, TM, and CNB. All authors listed have made a significant and direct contribution to this project and approved it for publication.

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1. Chapter 1: Introduction:

1.1. Introduction

Shiga-toxigenic *Escherichia coli* (STEC) are important enteric pathogens linked to serious foodborne illnesses outbreaks and economic losses involving meat and produce worldwide (Wang et al., 2016b; Wang et al., 2020). In 2019, 1462 cases of STEC infections were reported to the Canadian National Enteric Surveillance Program (NESP); approximately 27% were caused by O157 STEC and 73% of infections were caused by non-O157 (PHAC, 2020). As Ruminants are the main reservoir of STEC, STEC can contaminate meat by transferring from hide and feces to the carcasses during processing (Bryan et al., 2015; PHAC, 2015). In the USA (2013 – 2017), around 26% of STEC infections were attributed to beef, which is the second-highest estimated source in the USA except for Vegetable Row Crops (46%) (CDC, 2019a). To control the beef contamination with STEC in North America, federally registered beef packing plants are required to develop Hazard Analysis and Critical Control Point (HACCP) system accompanied with multiple pathogen reduction interventions (Gill, 2009; Yang et al., 2017a). Despite all the measures taken by the beef industry, STEC outbreaks caused by the consumption of contaminated beef products (CDC, 2016a; 2018; 2019c) showing that STEC control is a complex issue that needs more investigation. During beef processing, High Event Periods (HEPs) are observed sporadically, HEPs happen when a high number of beef carcasses or trimmings experience STEC contamination (Stanford et al., 2021), which becomes a challenge to the beef industry and public health and regulatory authorities. Due to food safety concerns related to HEP events, significant economic losses can occur related to beef product recall and destruction (FSIS, 2014; Wang et al., 2016b).

To reduce microbial contamination, some research has been carried out on trying to understand microbial contamination dynamics. Several studies have demonstrated that the generic *E. coli* isolated from contaminated beef trimmings seems mainly originated from beef fabrication equipment surfaces such as conveyor belts (Youssef et al., 2013; Yang et al., 2015). A study by Stanford et al. (2021) demonstrates more than 87% of *E. coli* isolates from beef processing

equipment were biofilm former, instead only 7% of *E. coli* isolated from cattle were capable of forming biofilm (Stanford et al., 2021). Despite only one out of 745 isolates from cattle being a strong biofilm former, they could be associated with HEP by surviving the sanitation process and being persistent on the beef fabrication equipment (Stanford et al., 2021). Biofilms are complex surface-associated bacterial assemblages (pathogenic and/or spoilage bacteria) composed of aggregates of sessile cells that are encased by an EPS matrix (Fratamico et al., 2009). Once biofilms are established, they act as shields, protecting bacteria that reside within the biofilm, preventing the penetration of sanitizers, and thus protecting the bacterial community that resides inside (Pang and Yuk, 2018). Mature biofilms make bacteria more difficult to remove, as well as pose the beef product under the risk of cross-contamination (Wang et al., 2014; Wang et al., 2016b). In addition, injured or nutrient-deprived cells have the opportunity to repair once embedded within a biofilm (Kumar and Anand, 1998b). Thus, robust biofilm former STEC more likely survived the sanitation process and became a continuous source of beef contamination on equipment surfaces (Stanford et al., 2021).

There are reports of STEC biofilms focused on single-species biofilms. However, these conditions might not represent the environment encountered by STEC on beef processing facilities. In meat processing plants, biofilms may be composed of bacteria with lactic acid bacteria (LAB), spoilage bacteria (SP), or pathogenic bacteria (Vogeleer et al., 2014; Wang et al., 2018; Visvalingam et al., 2019b). But little is known about the risk that these multispecies biofilms pose concerning beef contamination. Some members within biofilm communities may exhibit synergisms or antagonisms (Giaouris et al., 2014). Another aspect for biofilms to be considered is that they can be found in dry conditions (Adator et al., 2018). For example, biofilms can be formed under high humidity conditions, in the presence of organic material (e.g., meat juices, blood, water, etc.) during meat processing, which could facilitate bacterial growth, attached to conditioner layers surfaces and biofilm formation (Ma et al., 2020). However, after the plant is clean and surfaces are allowed to dry, biofilms that are not properly removed from food contact surfaces can remain on the surface, these biofilms dry out and can rehydrate and provide with nutrients once production

begins, posing a risk of food products cross-contamination (Adator et al., 2018). In Canada, beef processing plant maintained the environment temperatures lower than 10 °C to limit the growth of spoilage and enteric pathogens bacteria (Visvalingam et al., 2017a; Yang et al., 2017a; Yang et al., 2017b). However, the application of hot water during sanitation process on beef processing equipment may increase the surface temperature (Yang et al., 2017a; Wang et al., 2018). And thermoplastics and stainless steel are two of the most common material used in building food contact surfaces (Chia et al., 2009; Sofos and Geornaras, 2010). Limited information exists about STEC association with multispecies biofilms, thus more research is needed regarding their ability to form biofilms under different conditions (temperature, humidity, surface type, presence of other bacteria, etc.) and biofilm potential to cause beef contamination and persistence under desiccation conditions.

1.2. Research Hypothesis

We hypothesized that interspecies synergistic and antagonistic interactions may occurred within multispecies biofilm. And the STEC transfer to beef or persist within multispecies biofilm would be affected according to the temperature (10/ 25 °C), humidity (moist/ dry), contact surface (SS/ TPU) and aging days (6/ 30/ 60 d).

1.3. Research Objective

The main objectives of this study were to 1) evaluate potential synergistic and antagonistic interactions of STEC with either LAB or SP within multispecies biofilms formed on thermoplastic polyurethane (TPU) or stainless steel (SS); 2) determine the extent of transfer of STEC from single and multispecies biofilms to beef with different storage times, temperatures, and humidity and 3) determine the capacity of STEC to survive within single vs multispecies biofilms.

2. Chapter 2: Literature Review

2.1. Shiga-toxigenic *Escherichia coli* (STEC)

2.1.1. STEC General Information

Shiga-toxin-producing *Escherichia coli* (STEC) are *E. coli* that produce Shiga toxins and are important enteric pathogens worldwide. Shiga toxins secreted by STEC include both Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), which are separately encoded in two different Shiga toxin genes (*stx*1 & *stx*2) (Padola and Etcheverria, 2014).

The surface antigens are commonly used to classify STEC into specific subtypes (Bryan et al., 2015). The identification of each STEC subtype is commonly dependent on the O antigen (lipopolysaccharide somatic) and H antigen (flagellar); in addition, more comprehensive serotyping of STEC would require the K antigen (capsule) as extra information (Wolf, 1997; Whitfield, 2006; Bryan et al., 2015).

E. coli O157 was the first serotype identified and classified as STEC in the 1980s. (Karmali et al., 1983a; Bryan et al., 2015). Since then, a variety of non-O157 STEC serotypes has been reported, which are also capable of causing foodborne illness and lead to Hemolytic Uremic Syndrome (HUS) (Fratamico and Bagi, 2012). In North America, there are six non-O157 SETC serotypes identified by CDC as “top six” due to their association with foodborne disease outbreaks (Fratamico and Bagi, 2012). These six non-O157 serotypes include STEC O26, 045, O103, O121, O111, and O145 (Fratamico and Bagi, 2012).

2.1.2. STEC Virulence Factor

The infectious dose of STEC requires only ten cells to cause diseases in humans. STEC can cause severe foodborne illness and sometimes lead to serious complications such as Hemolytic Uremic Syndrome (HUS) and hemorrhagic colitis (Etcheverria et al., 2010; PHAC, 2015; Public Health Agency of Canada, 2015).

The STEC mechanism involves bacterial attachment to the large intestinal mucosa and the release of Shiga toxin which can cause epithelial cell death (Rahal et al., 2012). The Shiga toxin consists of two main subunits, A and B, as summarized by Rahal et al. 2012. The subunit B binds to the cell's surface receptors Globotriaosylceramide-3 (Gb3) and Globotetraosylceramide-4 (Gb4) allowing subunit A to enter into the epithelial cell (Rahal et al., 2012). Subunit A interrupts the protein synthesis by blocking the peptide elongation on the 60s ribosomal and leads to epithelial cell death (Hofmann, 1993). The epithelial cell death gives the Shiga toxin a chance to spread throughout the bloodstream and bind to the globotriaosylceramide-3 (Gb3), which is present in several organs (Nguyen and Sperandio, 2012). A relatively high amount of Gb3 is expressed by the human renal glomerular endothelium (Karmali et al., 1983b), which shows that in some cases the toxin can further spread through the bloodstream to infect other organs such as renal glomerular endothelium (Rahal et al., 2012). Since Gb3 is not expressed in cattle, ruminants are considered asymptomatic carriers (Karmali et al., 1983b).

As indicated by Byran et al. 2015, the toxic effect of Stx2 is more severe than Stx1. Only 8.4% of the patients infected by STEC producing Stx1 developed HUS (Bielaszewska et al., 2013; Bryan et al., 2015), whereas, 74% of the patients infected by STEC producing Stx2 developed HUS (Bielaszewska et al., 2013; Bryan et al., 2015). The Stx2 producing STEC strains were more prevalent than the Stx1 producing STEC (Etcheverria et al., 2010). It has been reported that STEC producing Stx2 contributes to 57.14% of the total isolates from the beef carcass, which is higher than the amount of STEC producing Stx1 at 9.52% and both Stx1 and Stx2 at 33.33% (Etcheverria et al., 2010).

Apart from Stx1 and Stx2, there are other virulence factors besides, such as the Locus of Enterocyte Effacement (LEE), and the Hemolysin A (*hly*A) operon which is encoded on the Bacterial pathogenicity islands (PAI) (Deng et al., 2004; Hussein and Sakuma, 2005; Rahal et al., 2012). In most STEC, all of the genes which are necessary to allow the attachment and effacing lesions of the human intestinal mucosa are located on the LEE (Vallance and Finlay, 2000; Bryan et al., 2015).

2.1.3. STEC Epidemiology

It is estimated that STEC contributes to 265,000 cases of foodborne illnesses annually in the USA, which include more than three-thousands hospitalization and thirty deaths (Scallan et al., 2011; Hale et al., 2012). About 474 confirmed cases of STEC O157 related foodborne diseases have been reported in Canada annually (PHAC, 2017). In the United States, the CDC has indicated that the chance of infection for either O157 and "top six" non-O157 serotypes are 0.66 and 0.72 cases for every one-hundred-thousand people respectively (CDC, 2016b). In Canada, O157:H7 was still the most common STEC serotype isolated from clinical cases (34.48%) in 2018 (Table 2.1), followed by O26:H11 (11.49%), O111:NM (5.75%), and O103:H2 (5.75%) (PHAC, 2019).

Ruminants are considered STEC's main reservoir (Bryan et al., 2015). Scientific data has shown that STEC can transfer from hide and feces to the carcasses during beef slaughtering (Bryan et al., 2015; PHAC, 2015), thus bovine-related products can be a source of STEC foodborne illness (Nguyen and Sperandio, 2012). The research by Stanford et al. 2016, found that 78.8 % of feces samples collected from cattle at Western Canadian slaughter cattle over two years, were positive for O157 serogroup (Stanford et al., 2016). The "top six" non-O157 subtype prevalence in cattle are listed as follows: O103 (94.4%), O45 (93.1%), O26 (82.3%), O121 (66.1%), O111 (8.2%), and O145 (7.0%) (Stanford et al., 2016). It has been reported that some STEC strains that can survive cleaning and sanitation can persist and serve as a source of cross-contamination in food processing facilities (Vogeleer et al., 2014). Contamination of beef products by the STEC could be also be caused by the formation of STEC biofilms on the various processing equipment in the beef processing facility (Vogeleer et al., 2014).

Table 2-1. The list of most frequent STEC subgroups causing foodborne illness (culture-confirmed) in Canada, 2018 (PHAC, 2019).

Rank	Serotype	Percentage
1	O157:H7	34.48
2	O26:H11	11.49
3	O111: NM	5.75
4	O103:H25	5.75
5	O121:H19	4.60
6	O26:NM	4.60
7	O103:H2	3.45
8	O145:NM	3.45

2.1.4. STEC Outbreak Related to Beef Commodities

As shown below in Table 2-2, the primary source of the beef related STEC outbreaks in North America is ground beef products and non-intact beef steaks. It has been reported that 40% of STEC foodborne illness outbreaks can be attributed to the consumption of undercooked ground beef (Smith et al., 2014). In North America, there is zero tolerance for STEC in ground beef (Ferrier and Buzby, 2014). In addition, non-intact steaks have been confirmed to have a significant role in STEC infections (Laine et al., 2005). Steaks that are blade tenderized and marinated through injection are being reported to have more risk of STEC contamination than non-tenderized and non-marinated steak (Laine et al., 2005). This is due to the effects of the blade tenderized and marinated injection, where the blades can translocate STEC from the surface of the beef cut to the interior, posing an additional risk if the beef is not well cook (Laine et al., 2005). Therefore, the blade tenderized steak should be cooked to achieve a 63°C internal temperature to eliminate STEC from inside of the steak (Currie et al., 2019). In 2012, a severe STEC O157:H7 outbreak was linked to the needle-tenderized beef steaks from Alberta, where around 4-thousand tons of beef products were recalled (Currie et al., 2019). Since then, it has been mandatory to label mechanically tenderized beef for identification accompanied by cooking instruction under Canada's Food and Drug Regulations (Currie et al., 2019). However, not all consumers cook the meat properly due to

their various cooking skills and consumption habits, so preventing STEC contamination during the beef fabrication process is necessary (Duffy et al., 2014).

Table 2-2. STEC outbreaks related to bovine products in North America since 2010.

Country	Year	STEC serotype	Bovine products	Cases (deaths)	HUS	References
Canada &USA	2019	O103 & O121	Ground Bison	33	0	(CDC, 2019b)
USA	2019	O103	Ground Beef	209	2	(CDC, 2019c)
USA	2018	O26	Ground Beef	18 (1)	1	(CDC, 2018)
USA	2016	O157: H7	Beef, Veal, Bison Product	11	1	(CDC, 2016a)
USA	2014	O157: H7	Ground Beef	12	0	(CDC, 2014b)
USA	2010	O157: H7	Non-intact Steaks	21	1	(CDC, 2010)

2.2. Cattle Processing Procedure and the Processing Environment in Beef Industry

2.2.1. Beef Processing and STEC Contamination

The processing of the cattle can be divided into “live animal procedure” and “slaughter process” (Galland, 1997). Once the cattle have been purchased from the farm, they would be allocated into each holding pen for a few hours to days based on their designated lot number before the slaughtering process (Galland, 1997). After the cattle have been stunned, they are hung by one of the hind legs for de-hiding and skinning processes (Etcheverria et al., 2010). Some slaughter facilities do hide washing to remove contamination before slaughter, which can efficiently reduce the prevalence of *E. coli* O157:H7 and *Salmonella* on the beef hide (Arthur et al., 2007). After the evisceration process, the carcass is subject to “multiple antimicrobial interventions” and enters the cooler for chilling to 7 °C (Arthur et al., 2014; Visvalingam et al., 2017a). Before the final fabrication process, the whole carcass is chilled down and graded (Arthur et al., 2014). After the primary and sub-primary cutting, the designated carcass section continues with processing to turn

into the specific beef products (Arthur et al., 2014).

Beef carcasses contaminated by STEC can be primarily attributed to the hide and fecal material during the dressing and slaughtering processes (Etcheverria et al., 2010). The STEC contamination levels in the cattle feces and hides have been positively correlated with STEC contamination conditions in the slaughtered beef carcass (Byrne et al., 2000; Elder et al., 2000). In addition, the more processed beef products, especially cutting and chopping, tend to have a higher risk of STEC contamination (Etcheverria et al., 2010). As indicated by Etcheverría et al. in 2010, the percentage of ground beef positive to STEC (40.74%) was significantly higher than STEC contamination on whole beef cuts rump roast cuts (12.12%), and beef chuck (12.12%) (Etcheverria et al., 2010). Scientists attribute this elevated contamination to the grinding process, where STEC present on some beef cuts is ground and mixed with non-contaminated beef and therefore STEC can be spread and compromised the whole ground beef bin. After grinding the meat also offers more surface area for STEC colonization (Duffy et al., 2006; Duffy et al., 2014).

2.2.2. Pre-harvest and Post-harvest Control Strategy

To prevent or reduce the STEC and other foodborne pathogens' risk of contamination during beef processing, antimicrobial interventions at pre-and post-harvest levels have been developed. Currently, pre-harvest control methods for reducing the STEC within the cattle's gastrointestinal tract are still under investigation, which includes but are not limited to adding probiotic and antimicrobial agents into the feed (Sargeant et al., 2007b; Wheeler et al., 2014). For example, some studies demonstrated *Lactobacillus* spp. can reduce the shedding of *E. coli* O157:H7 in cattle feces (Brashears et al., 2003; Younts-Dahl et al., 2004). The post-harvest control of the STEC based on the hurdle-technology concept is the primary intervention currently applied in the beef industry, which includes the application of both chemical and physical interventions at the beef processing facility (Koohmaraie et al., 2005). As mentioned by Duffy et al. in 2006, the carcass after the dressing process, will experience the first round of the decontamination methods, which includes the trimming of "visibly dirty areas", and the steam vacuuming followed by washing (1.5% NaOH)

(Duffy et al., 2006). The second-round decontamination methods include hot water washing (74 °C more than 5 seconds) followed by steam pasteurization (Koohmaraie et al., 2005; Duffy et al., 2006). The combination of those two rounds of decontamination methods is estimated to contribute to approximately a 1.5 $\log_{10}$ reduction of the *E.coli* O157: H7 on beef carcasses (Duffy et al., 2006).

The third-round interventions included the application of chlorine (Cl), chlorine dioxide (ClO_2), acidified sodium chlorite ($NaClO_2$), trisodium phosphate (Na_3PO_4), activated lactoferrin (ALF), and organic acids (e.g. lactic acid) on the beef carcasses (Dorsa et al., 1997; Gill and Badoni, 2004; Koohmaraie et al., 2005). Research has shown that spraying or dipping the *E. coli* O157 contaminated beef carcass into a lactic acid solution (2% - 5%) can reduce O157 from 5 logs to 2 $\log_{10}$ CFU/cm^2 (King et al., 2005). After processing, it is important to maintain a proper refrigeration temperature (4 °C) during the beef product storage (e.g. ground beef) to inhibit the recovery and growth of STEC.

2.2.3. Cleaning and Disinfection Technologies in the Beef Industry

In addition to pre and post-harvest control, the proper implementation of the regular cleaning and disinfection process is essential for maintaining the wholeness and food safety of the final products; especially to prevent cross-contamination (Gibson et al., 1999). When food safety programs are not properly implemented and followed, out-of-control conditions can happen, including failing to fully comply with cleaning and disinfection procedures, especially during meat fabrication where contaminated meat pieces (e.g. STEC) can spread bacteria to the food contact surface during the fabrication process, and further contaminate other meat products (Gibson et al., 1999; Gill et al., 1999).

The cleaning and disinfection methods in the meat industry can be divided into four steps. First, the physical removal of organic material and soil from food contact surfaces then the application of hot water (45 °C) to rinse the food contact surface (Khamisse et al., 2012). Second, a 20-minute soaking is implemented to break down the soil structure and weaken its attachment on the food contact surface by the applications of detergents such as chlorinated alkaline solution

accompany with mechanical removal (Gibson et al., 1999; Khamisse et al., 2012). Third, is the application of water to remove detergents (Khamisse et al., 2012). Fourth, food contact surfaces are cleaned and sanitized. Some of the common food-grade sanitizers include but are not limited to quaternary ammonium and glutaraldehyde (Khamisse et al., 2012).

2.2.4. Common Microflora Identified in Beef Industry

Hultman et al. in 2015, reported that there is a wide diversity of cold tolerance spoilage bacteria in meatpacking plants, which include but are not limited to *Leuconostoc*, *Aerococcus*, *Carnobacterium*, *Lactobacillus*, *Pediococcus*, *Pseudomonas*, and *Yersinia* (Hultman et al., 2015). Previous studies demonstrated multiple bacteria can continually be present on the beef fabrication equipment after the sanitation process (Khamisse et al., 2012; Wang et al., 2018). For example, *Yersinia*, *Serratia*, and *Raoultella* were the three major *Enterobacteriaceae* isolated from the sanitized non-food contact surface before production (Wang et al., 2018). In the same study, *Carnobacterium* was identified as the only lactic acid bacteria that persisted on non-food contact surfaces after sanitation (Wang et al., 2018). Interestingly, *Carnobacterium* was also observed as predominant genera among the microflora of refrigerated vacuum-packaged beef from the same beef fabrication plant (Youssef et al., 2014). More information regarding other LAB and spoilage bacteria genus and species that has been isolated from different beef products is summarized in Table 2-3.

Table 2-3. Common Lab and spoilage bacteria identified from refrigerated beef products.

Spoilage bacteria	Source	Storage condition	Reference
Leuconostoc gelidum	vacuum-packed beef product	2 °C/ 6 weeks	(Sakala et al., 2002)
Leuconostoc mesenteroides	vacuum-packed beef product	2 °C/ 6 weeks	(Sakala et al., 2002)
Carnobacterium divergens	vacuum-packed beef product	2 °C/ 160 days	(Youssef et al., 2014)
Carnobacterium piscicola	vacuum-packed beef product	2 °C/ 6 weeks	(Sakala et al., 2002)
Carnobacterium maltaromaticum	vacuum-packed beef product	2 °C/ 160 days	(Youssef et al., 2014)
Lactobacillus sakei	vacuum-packed beef product	2 °C/ 6 weeks	(Sakala et al., 2002)
Lactobacillus curvatus	vacuum-packed beef product	2 °C/ 6 weeks	(Sakala et al., 2002)
Hafnia alvei	Beefsteak	2 °C/ 2 weeks	(Ercolini et al., 2006)
Rahnella alvei	Beefsteak	2 °C/ 2 weeks	(Ercolini et al., 2006)
Serratia proteamaculans	Beefsteak	2 °C/ 2 weeks	(Ercolini et al., 2006)

2.3. STEC Biofilm Formation Mechanisms in Beef Processing Facility

2.3.1. Biofilm General Information

Biofilm can be defined as a microorganisms community attached to a solid surface or to each other, covered within a self-produced Extracellular Polymeric Substance (EPS) which acts as cement keeping microorganisms attached to a solid surface or to each other (Srey et al., 2013; Vogeleer et al., 2014; Adator et al., 2018). In a biofilm, EPS are commonly produced to facilitate bacteria aggregation and biofilm formation, and the major component of EPS include but are not limited to curli fibers, colonic acid, cellulose, and the poly-N-acetylglucosamine (Wang et al., 2013b; Vogeleer et al., 2014). Pathogenic bacteria (e.g. STEC), spoilage bacteria, and lactic acid bacteria can form multispecies biofilm on food contact surfaces (Sofos and Geornaras, 2010). Furthermore, the competitive and cooperative interaction among different microorganisms can be observed among different species within the multispecies biofilm (Yang et al., 2011). In present studies, the bacteria species that can produce lactic acid were classified as LAB bacteria to

investigate their interaction with STEC, even though some LAB bacteria such as *Carnobacterium* can also cause food spoilage. (Leisner et al., 2007).

2.3.2. Biofilm Formation Stages

Biofilm development takes place in four stages: 1) reversible attachment, 2) irreversible attachment, 3) biofilm development and maturation, and 4) dispersion or detachment (Srey et al., 2013). Bacteria, including STEC, can form biofilms on food contact surfaces facilitated by inappropriate cleaning and sanitation (Kumar and Anand, 1998a; Wang et al., 2012b).

2.3.2.1. Reversible Attachment

The planktonic cells can be reversibly attached to the food contact surface as the first step of the biofilm formation, marked by either Brownian motion (passive) and bacteria motility (active) (Kumar and Anand, 1998a; Van Houdt and Michiels, 2005a). Weak interactions are maintained in the reversible attachment between the planktonic cell surface appendages (e.g. flagella) and the food contact surfaces, which can include a hydrophobic effect, electrostatic charge, and Van Der Walls attractive forces (Kumar and Anand, 1998a).

2.3.2.2. Irreversible Attachment

Under the assistance of bacteria self-producing EPS (e.g. curli), the attachment becomes irreversible due to the permanent connection formed between the food contact surface and the attached cell (Kumar and Anand, 1998a; Srey et al., 2013; Vogeleer et al., 2014).

2.3.2.3. Biofilm Maturation

Mature biofilms exhibit a mushroom-shaped structure formed by a multilayer of bacteria cells

mixed with the EPS. Water channels are also formed, to spread nutrients throughout the whole biofilm, and help to channel and remove the waste from the biofilm. (Kumar and Anand, 1998a; Srey et al., 2013).

2.3.2.4. Dispersion

Upon biofilm maturation, bacteria within the biofilm detaches, at this stage as planktonic cells, then the bacterial cells spread colonizing other surfaces, in the context of food processing environments, acting as a source of cross-contamination (Vogeleer et al., 2014). The mechanism causing the biofilm detachment is complex. It has been reported that the biofilms detachment mechanism could include shear forces, for instance when the biofilm comes into contact with food (passive) (Srey et al., 2013; Vogeleer et al., 2014). Another detachment theory is attributed to EPS degradation due to the enzymatic action (Srey et al., 2013; Vogeleer et al., 2014). For example, bacteria can actively release polysaccharide enzymes for EPS degradation when the bacteria are stressed due to environmental changes (Vogeleer et al., 2014). STEC strains left behind in the food processing environment due to improper sanitation can form biofilms or shelter within pre-existing biofilms (Srey et al., 2013; Vogeleer et al., 2014). Eventually, cells from mature biofilms can detach and become a continual source of cross-contamination (Kumar and Anand, 1998a; Wang et al., 2012b).

2.3.3. Biofilm Growth Condition in Beef Industry

In the food industry, two of the most common materials used to build food processing equipment are stainless steel and plastic (polyurethane), both hydrophobic and thus able to support biofilm formation (Chia et al., 2009; Sofos and Geornaras, 2010). Researchers report that bacteria (e.g., *Salmonella* & *Listeria*) attach to the more hydrophobic surfaces (plastic) not only in higher numbers but also more rapidly than to the less hydrophobic material (metal) (Sinde and Carballo, 2000; Donlan, 2002). The authors hypothesized that hydrophobic interactions between the cell and

the hydrophobic surface can help the cells overcome repulsive forces associated with the contact surface during the cell attachment (Sinde and Carballo, 2000; Donlan, 2002). *E. coli* O157: H7 and *Listeria monocytogenes* can develop stronger monoculture biofilms (higher bacteria count) on the plastic surfaces (e.g. polyurethanes) than on the stainless steel surfaces (SS-304) (Midelet and Carpentier, 2002; Graziella et al., 2006; Sofos and Geornaras, 2010).

Food contact surfaces are required to be cleaned and sanitized daily in the food industry, however, despite the cleaning and sanitation, bacterial biofilms might be able to develop on moist non-food contact surfaces (e.g. ceiling & wall) due to the infrequent cleaning and sanitation (Chmielewski and Frank, 2003) and indirectly cross-contaminate food contact surfaces, for example through food handlers. The meat debris splashed during the beef processing can help the bacteria to attach and grow on both food and non-food contact surfaces, which could lead to biofilm formation (Sofos and Geornaras, 2010; Srey et al., 2013). The organic material exuded from the beef carcasses or beef cuts, such as beef purge, can serve as a conditioning layer on the solid surface (e.g. stainless steel), which can also facilitate the attachment of bacteria (Tang et al., 2009). The growth rate of *E. coli* O157: H7 is slower on the dried stainless-steel surfaces than on the wet ones due to lower nutrients and water availability (Sofos and Geornaras, 2010). However, a stronger attachment is demonstrated on the dry stainless-steel surface than the wet surface (Sofos and Geornaras, 2010). Regarding temperature effects on biofilm formation, an experiment conducted by Dourou et al. in 2011 showed that STEC O157:H7 was able to attach on stainless steel and plastic conveyor belts under beef storage temperatures (4 °C), and further multiply under beef processing temperature (15 °C) (Dourou et al., 2011; Fouladkhah et al., 2013; Srey et al., 2013; Galie et al., 2018).

Hot water (40 -50 °C) is commonly applied to the food contact surface to remove beef debris for beef fabrication facilities (Wang et al., 2018). As reported by Wang et al. in 2018, the population of the mesophilic bacteria (e.g. *Stenotrophomonas*) predominant on the sanitized conveyor belt before the beginning of production, indicating that the surface temperature likely increased after sanitation to support the mesophilic bacterial growth (Wang et al., 2018). Also, if the water is too

hot it could aid protein fixation to the surfaces, which will also favor the conditioner layer and bacterial contamination.

A study conducted by Visvalingam et al. in 2019, pointed out the importance of using bacteria naturally present in the beef processing facilities when studying multispecies biofilms. Bacteria isolated from the meat processing environment can adapt to stressful conditions (post-harvest intervention) during beef processing and compete with other strains in the multispecies biofilm. Data collected using bacteria naturally present in the beef processing facility to investigate STEC biofilm formation may be more applicable to the reality of the meat industry (Visvalingam et al., 2019a).

2.3.4. Factors Influencing Biofilm Forming Ability and Bacteria Persistence

Biofilm-forming ability varies across bacterial strains, and it is influenced by nutrient condition, temperature, pH level, and the contact surface characteristics (Donlan, 2002; Wang et al., 2012a; Srey et al., 2013; Adator et al., 2018). Those factors could affect biofilm development during the different steps and alter the structure and functional property of the mature biofilm ineluctably (Vogeleer et al., 2014). Besides, the polysaccharides, curli production, and intercellular communication (Quorum Sensing) have been reported to play a significant role in STEC biofilm-forming ability and persistence (Wang, 2019).

2.3.4.1. Exopolysaccharides Production

The polysaccharides are one of the essential components of the EPS matrix. EPS is secreted by the bacterial community and it can surround each bacterial cell, forming a complex matrix around the bacterial community (Flemming and Wingender, 2010). As summarized by Flemming et al. in 2010, the exopolysaccharide participating in biofilm formation can be classified into homopolysaccharide (e.g. cellulose) and heteropolysaccharide (e.g. colonic acid) (Flemming and Wingender, 2010). The heteropolysaccharide carries the organic or inorganic substituents, which

are believed to have a significant effect on its chemical and physical characteristics (Flemming and Wingender, 2010). The production of exopolysaccharides is essential for the bacteria's biofilm-forming ability; however, the exopolysaccharides composition is strain-dependent (Ma et al., 2009). For example, at least three different kinds of exopolysaccharides have been produced by *P. aeruginosa* during the biofilm formation, which includes Pel, Psl, and alginate (Ryder et al., 2007; Byrd et al., 2009; Ma et al., 2009). The alginate plays a significant role in the early stage of biofilm formation (Ryder et al., 2007). The Pel facilitates biofilm formation on both the solid surface and liquid-air interface (Ryder et al., 2007; Byrd et al., 2009). The Psl is important in attachment on the solid surface and maintaining the biofilm structure (Ryder et al., 2007; Byrd et al., 2009).

2.3.4.2. Curli and cellulose production

Curli fibers are defined as "long, thin, flexible proteinaceous filaments," which are built by CsgA (major) and CsgB (minor) subunits (Van Houdt and Michiels, 2005b). As per a review by Barnhart et al. 2006, curli plays a significant role in enteric bacteria's (*E. coli* & *Salmonella* spp.) surface and cell to cell contact (Barnhart and Chapman, 2006). Previous studies indicated that curli production can facilitate biofilm formation on abiotic surfaces and community behavior (Van Houdt and Michiels, 2005b; Barnhart and Chapman, 2006). For example, curli producing *E. coli* O157:H7 strains can better form biofilm on stainless steel surfaces when compared to the weak-curli producing strains (Ryu et al., 2004). Visvalingam et al. 2017 noticed that all curli and cellulose positive *E. coli* strains showed stronger biofilm-forming ability in microtiter plates than non-curli and cellulose producers (Visvalingam et al., 2017a). During biofilm development, curli fibers can associate with the cellulose to form a hydrophobic polymer (EPS); therefore, STEC strains thar are able to express curli, often correlate with a thicker biofilm formation and a higher persistence phenomenon (Gualdi et al., 2008; Iibuchi et al., 2010; Adator et al., 2018). For instance, the hydrated EPS matrix covers the biofilm-like gel to protect the bacteria against desiccation (Kumar and Anand, 1998a; Stewart and Franklin, 2008). The EPS keeps the bacteria in a moist environment

due to the high water-keeping capacity of the EPS (Kumar and Anand, 1998a; Stewart and Franklin, 2008). EPS can retard the biofilm dehydration and trap nutrients that are available to provide nutrients to the bacterial community within the biofilm (Kumar and Anand, 1998a; Stewart and Franklin, 2008). In addition, persistent STEC cells are more likely to survive the disinfection process when associated with a biofilm. The EPS matrix can also act as a shield, thus reducing the STEC cell contact with the sanitizer (Fouladkhah et al., 2013; Vogeleer et al., 2014). The EPS substance may also react with the antimicrobial chemicals diffusing into the biofilm. Some of the reaction mechanisms include but are not limited to chelation, enzymatic degradation, and oxidizing the sanitizer (Daddi Oubekka et al., 2012; Flemming et al., 2016).

2.3.4.3. Intercellular Communication - Quorum Sensing

During biofilm development, the gene expression of the bacteria within the biofilm varies according to the biofilm stages of development (Whiteley et al., 2001). Variations in gene expression are regulated under cell-to-cell communication, known as Quorum Sensing (QS) (Walters and Sperandio). For example, the QS signal, including N-acyl-homoserine lactones (AI-1) and furanose borate diester (AI-2), could accumulate within the bacteria aggregate (Wells, 2022). Since *E. coli* can sense the AI-1 signal, the accumulation of AI-1 in the biofilm can cause upregulation of *uvrY* and *csrA* to enhance biomass production (Wells, 2022). The QS system's mechanism to alter the gene expression in STEC biofilm is complex and can be affected by environmental factors such as temperature and nutrient content. (Silagyi et al., 2009; Vogeleer et al., 2014). For instance, some research has shown that when complex nutrients such as beef purge are supplemented in the culture media, STEC would release AI-2 to alter the expression of the gene, to induce colonic acid production, and enhance the biofilm formation (Lu et al., 2005; Silagyi et al., 2009). The AI-2 was a non-specific signal produced by S-ribosylhomocysteine lyase (LuxS), which has been used by both gram-positive and negative bacteria (Sargeant et al., 2007a).

Previous studies indicated that QS systems also seem enhanced multispecies biofilm sanitizer tolerance through providing interspecies communication (Wang, 2019). For example, Kong et al.

(2017) demonstrated that a QS signal (Farnesol) produced by *Candida albicans* could significantly enhance the antimicrobial resistance of *Staphylococcus aureus* in their dual-species biofilm because the upregulation of drug efflux pumps activity of *S. aureus* was triggered by farnesol-induced oxidative stress (Kong et al., 2017).

2.3.5. Cleaning and Sanitation Technology Applied to Remove Biofilm

A study conducted by Gibson et al. 1999, showed that the cleaning process was able to eliminate more than 0.9 $\log_{10}$ (CFU/ swab) of the bacteria from the stainless steel, and the subsequent sanitation process could bring further reduction of at least 1 $\log_{10}$ (CFU/ swab) (Gibson et al., 1999). However, persistent STEC is more likely to survive the disinfection process under the protection of biofilms, which entrap STEC within the EPS and thus circumventing the contact with the sanitizer (Fouladkhah et al., 2013; Vogeleer et al., 2014).

In the beef industry, some bacteria such as generic *E. coli* have been reported to persistently exist in the beef processing environments, for example on the conveyor belt, and might become a reoccurring source of cross-contamination (Yang et al., 2018). As reported by Yang et al. 2018, more than 80% of *E. coli*, which were isolated from beef fabrication equipment, were able to form strong biofilms on day 6 and survive after QAC (200 ppm) treatment (Yang et al., 2018). A similar result has been observed by Wang et al. 2012, who noticed the STEC bacteria at the biofilm stage after 72 h incubation on polystyrene microplate showing more sanitizer tolerance than its planktonic stage (Wang et al., 2012a). Meanwhile, the curli and cellulose (EPS) produced during the 72 h STEC biofilm development in the polystyrene microplate was believed to play a significant role in biofilm formation and sanitizer tolerance (Wang et al., 2012a; Wang et al., 2013b). Therefore, the best time to control biofilm is to remove the planktonic cells from the food contact surface before the mature biofilm develops (Midelet and Carpentier, 2004; Srey et al., 2013). When already targeting formed biofilms, the cleaning process must break the biofilm EPS shield through mechanical forces (i.e. brushes) to expose the bacteria to the sanitizer and to remove the bacteria efficiently (Chmielewski and Frank, 2003).

Sanitizer commonly applied in the food industry to remove biofilm includes but is not limited to chlorine, quaternary ammonium compounds (QAC), hydrogen peroxide (H_2O_2), and peroxyacetic acid (Chmielewski and Frank, 2003). The mechanisms of action vary with each sanitizer. Chlorine possesses a strong oxidizing ability but it can lose its antimicrobial properties when it is in contact with organic material (Chmielewski and Frank, 2003). The QAC can cause damage to bacteria's cytoplasmic membrane by protein denaturation, which will cause bacteria cell death (Denyer and Stewart, 1998). Peroxygen sanitizers, which include H_2O_2 and peroxyacetic acid, can inactivate bacteria cells by damaging the sulfur (S-S) and sulfhydryl (S-H) bond on the protein and enzymes to increase cell wall permeability (McDonnell and Russell, 1999). As indicated by McDonnell et al. in 1999, the peroxyacetic acid is more potent biocide when compared with H_2O_2, and able to maintain the auctionability when in contact with organic material (McDonnell and Russell, 1999). EDTA (chelating agents) can be applied to break down the biofilm EPS matrix and expose bacteria to antimicrobials (Kumar and Anand, 1998a).

2.4. High Event Periods (HEP) and *E. coli* Persistency in Beef Industry

2.4.1. Definition and Regulation of HEP

The definition provided by Food Safety and Inspection Service (FSIS) in 2014 is as follows, "High event periods (HEP) are periods in which slaughter establishments experience a high rate of positive results for STEC (or virulence markers) in trim samples from production lots containing the same source materials" (FSIS, 2014). HEP can compromise beef carcass safety and the subsequently related beef products (FSIS, 2014). More than 80% of STEC coming from HEP trimmed beef products are identified as closely related to human illness (Arthur et al., 2014). Due to food safety concerns related to HEP events, significant economic losses can occur related to beef product recall and destruction (FSIS, 2014; Wang et al., 2016a; Wang et al., 2016b).

2.4.2. Relationship between HEP and STEC Biofilm Formation

High Event Periods are often attributed to fecal and hide contamination during the beef slaughtering process (Byrne et al., 2000; Elder et al., 2000). Therefore, the genomic diversity of STEC strains isolated from the HEP are expected to be consistent with the genomic diversity of STEC on beef fecal and hides (Byrne et al., 2000; Elder et al., 2000; Arthur et al., 2014). However, this is not the case. Previous research has shown that only one predominant STEC strain has been isolated from each individual HEP in large beef processing facilities (Arthur et al., 2014; Visvalingam et al., 2016). This finding is contrary to the conventional hypothesis that HEP is caused by multiple STEC strains (genomic diversity) on the beef fecal and hide (Arthur et al., 2014; Visvalingam et al., 2016). It has been reported that the *E. coli* genotype isolated from beef trimmings was consistent with the *E. coli* genotype isolated from the beef fabrication equipment surface instead of the beef carcass (Yang et al., 2015; Yang et al., 2017a). Therefore, the beef fabrication equipment surface such as conveyor belts (plastic) and cutting tables (stainless steel) are believed to act as the major source of *E. coli* contamination for beef products (Yang et al., 2017a). Yang et al. 2018, reported that *E. coli* biofilms on food contact surfaces might contribute to the *E. coli* persistence on the beef fabrication equipment (Yang et al., 2018). Moreover, it has been demonstrated that STEC strains (e.g. *E. coli* O157: H7) isolated from HEP beef products have strong biofilm-forming ability and resistance toward sanitizers (Wang et al., 2014). Therefore, STEC forming biofilm and persistence might play a significant role in the HEP phenomenon (Wang et al., 2014).

2.5. Spoilage and Lactic Acid Bacteria and its Synergistic and Antagonistic Effects on STEC Biofilm Formation and STEC Persistence

2.5.1. Multispecies Biofilm

Multispecies biofilms are coordinated by the interaction of different microbial species;

therefore, both competitive and cooperative interactions can happen (Stewart and Franklin, 2008; Rendueles and Ghigo, 2012). In research conducted by Burmolle et al. 2014, the functional property of the multispecies biofilm can differ from that shown in single-species biofilm (Burmolle et al., 2014). Therefore, applying the knowledge learned from the single-species biofilm to explain the property of the multispecies biofilm could be inaccurate (Burmolle et al., 2014; Rendueles and Ghigo, 2015).

In nature as well as in the food processing environment, bacteria are often found forming multispecies biofilms. Thus, to understand bacterial persistence, more information is needed to improve current risk assessment and to develop efficient pathogen reduction interventions or to assess food safety current practices. Thus to fill information gaps, it is important to investigate the interactions between pathogenic bacteria such as STEC and other bacterial strains commonly found in food processing environments (e.g. spoilage bacteria) regarding biofilm formation abilities (Giaouris et al., 2015).

2.5.2. Synergistic Effects in Multispecies Biofilm Formation

The synergetic interactions among the multispecies biofilms can result in beneficial outcomes for the whole bacteria community (Elias and Banin, 2012). For example, synergetic interactions have been reported to result in higher bacterial counts relative to single-species biofilm (Giaouris et al., 2015). Meanwhile, the synergistic effect is also shown as the higher biomass produced and antimicrobial tolerance by the multispecies biofilm than by either single-species biofilm (Burmolle et al., 2014; Giaouris et al., 2015). In the case of synergistic interactions within the multispecies biofilm, the environment within the biofilm should be suitable for all the members to grow. In addition, each strain benefits from the intermediate or end metabolic products of other bacterial strains (Giaouris et al., 2015).

As indicated by Liu et al. in 2014, a multispecies biofilm can be formed by *E. coli* O157: H7 and other biofilm formers such as *Ralstonia insidiosa*. A synergistic effect is attributed to a higher O157 cell count and thicker biofilm (Liu et al., 2014). In addition, previous research investigating

the ability of *E. coli* (PHL565) to form multispecies biofilms with either *Pseudomonas putida* (MT2) or *Staphylococcus epidermidis* (ATCC-155), and the results showed significant synergistic effect only in the multispecies biofilms composed of *E. coli* (PHL565) and *Pseudomonas putida* (MT2) when compared with *E. coli* (PHL565) single-species biofilm (Castonguay et al., 2006). Therefore, it has been shown that the synergistic effect within the multispecies biofilm varies among participating bacterial species and strains (Castonguay et al., 2006; Liu et al., 2014).

Visvalingam et al. in 2019 reported synergistic interactions within dual-species biofilms formed by *Salmonella Typhimurium* and *Pseudomonas helvolus*, where extra biomass (EPS) production was found (Visvalingam et al., 2019b). It has been reported that with synergetic interactions, the extra EPS (e.g. curli) produced by multispecies biofilm can act as a protective barrier for the whole bacterial community residing in the same biofilm against environmental stress and antimicrobial agents including sanitizers (Mah and O'Toole, 2001; Wang et al., 2013b).

A better understanding of the synergistic interactions among bacterial strains within the multispecies biofilm is needed to decipher STEC persistence in the beef industry (Rendueles et al., 2011; Jahid and Ha, 2014; Wang et al., 2014). Some aspects such as antimicrobial resistance and tolerance to chemicals used for sanitization have been studied and reported but research related to STEC bacterial persistence in the multispecies biofilm during long period storage within the desiccation environment is needed (Burmolle et al., 2014).

2.5.3. Antagonism Effect in Multispecies Biofilm Formation

Antagonistic interactions in multispecies biofilms are commonly associated with lower biomass production (e.g. EPS) and a decrease in the bacterial count of one or more participating species when compared with single-species biofilm (Visvalingam et al., 2017a). It has been reported that the synergistic interactions that happen at the beginning of the multispecies biofilm development can shift to an antagonistic effect during the long period of incubation. For example, the dual-species biofilm formed by *Salmonella Typhimurium* was initially showing synergistic (day 2) but turned to antagonistic with either *Aeromonas* (day 4), *Comamonas* (day 6), or

Pseudomonas (day 6) (Visvalingam et al., 2019b).

The best-described mechanism of the interspecies competition within the multispecies biofilm includes:

1) nutrients deficient and releasing an antimicrobial product,

2) solid surface hydrophobicity alteration,

3) gene expression alteration (Rendueles and Ghigo, 2012; Rendueles and Ghigo, 2015).

2.5.3.1. Nutrients Deficient and Releasing Antimicrobial Product

Certain bacterial species outcompete other species within the multispecies biofilm by either better seizing nutrients or by releasing a harmful metabolism product (e.g. organic acid) or antimicrobial peptides (e.g. bacteriocin) to inhibit the growth of other bacterial species (Wuertz et al., 2004; Rendueles and Ghigo, 2012). Previous research comparing multispecies (*E. coli* O157:H7 plus generic *E. coli* 136) and single species *E. coli* O157 biofilms has shown an *E. coli* O157 reduction of up to 1.5 $\log_{10}$ CFU can occur within the mature multispecies biofilm (Visvalingam et al., 2017a). The antagonism phenomenon between the generic *E. coli* and O157 was attributed to the competition for the nutrients and the toxic effect of the generic *E. coli* release of bacteriocin (e.g. colicin) against O157 (McAllister et al., 2011; Visvalingam et al., 2017a).

2.5.3.2. Solid Surface Hydrophobicity Alteration

As summarized in section 2.3.3, both Gram-positive and Gram-negative bacteria can attach to the more hydrophobic surface (plastic) in higher numbers and faster than when compared to the less hydrophobic material (metal & glass) (Sinde and Carballo, 2000; Donlan, 2002). It has also been reported that *E. coli* (e.g. Ec300) released a high molecular weight soluble polysaccharide after biofilm maturation, which significantly increases the hydrophilicity of the plastic surface, which antagonize the attachment and the biofilm formation of the gram-positive bacteria (e.g. *Staphylococcus aureus*) on the plastic surface (Rendueles et al., 2011). Similarly, another study

demonstrated that a soluble polysaccharide substance released by *E. coli* can affect the hydrophobicity of the glass surface and reduce the bacteria attachment and biofilm-forming abilities of both gram-positive and gram-negative bacteria on the glass surface (Valle et al., 2006b).

2.5.3.3. Gene Expression and Quorum Sensing

Quorum sensing (QS) is defined as intercellular communication by producing, receiving, and responding to signal agent (Li and Tian, 2012). QS is believed to play a significant role in the antagonistic interactions within the multispecies biofilm (Rendueles and Ghigo, 2015). As summarized by Lopes et al. in 2011, bacteria metabolites such as various toxins, antibiotics, some metabolic byproducts, and other signal substances have a role in the biofilm QS interaction (Lopes et al., 2011). Those metabolites are produced and accumulated within the biofilm during bacteria growth, and they can alter the nearby bacteria's gene expression and further lead to the bacteria's physiology change (Parsek and Greenberg, 2005; Lopes et al., 2011). Recent research indicates that a substance (e.g. released exopolysaccharide) produced by lactic acid bacteria can potentially be used as a biocontrol method to decrease STEC persistence by interfering with its biofilm formation (Valle et al., 2006a; Kim et al., 2009). Specifically, O157 surface adhesins (curli) expression can be inhibited by the lactic acid bacteria (*Lactobacillus acidophilus*) releasing exopolysaccharides (Kim et al., 2009). The expressions of the curli-related genes such as *csgA* and *csgB* were inhibited significantly, and there was no effect observed on the growth rate of the O157 (Kim et al., 2009). Similar studies conducted by Wang et al. in 2013, showed that the acyl-homoserine lactones (AHLs) produced by *P. aeruginosa* cannot only inhibit the *Salmonella* growth during the exponential phase but also interferes with its biofilm-forming ability (Wang et al., 2013a). Even though *Salmonella* doesn't carry the AHLs producing gene, the AHLs receptor (sdiA) makes *Salmonella* able to receive the AHLs signal released from *P. aeruginosa* (Soares and Ahmer, 2011). Receiving the AHLs is believed to decrease the *Salmonella* EPS production and compromise its ability to adhere to a solid surface (Wang et al., 2013a). Also, the AHLs synthesized by *P. aeruginosa* were able to disturb the *E. coli* biofilm-forming ability (Lee et al., 2007).

2.5.4. Factors Influencing Multispecies Biofilm development

Besides the synergistic and antagonistic interactions, some other factors can influence the interspecies interaction during the multispecies biofilm development, which includes but is not limited to 1) chemical heterogeneity within the biofilm, 2) bacteria colonization sequence, and 3) bacteria culture inoculum concentration.

2.5.4.1. Chemical Heterogeneity within Biofilm

Chemical heterogeneity refers to a chemical (e.g., oxygen) concentration gradient of the metabolic substrates and products within the mature biofilm (Rendueles and Ghigo, 2015). For example, the bacterial cells located deeper within the biofilm will experience lower oxygen concentration, more nutrient deficiency, and concentrated waste products than out surface (Rendueles and Ghigo, 2015). Some researchers have shown that due to the chemical heterogeneity within the biofilm, different bacterial groups will thrive in the different microenvironments within the biofilm. For example, differences in oxygen distribution will favor the multiplication of facultative anaerobic bacteria (Stewart and Franklin, 2008). Visvalingam et al. (2019) demonstrated that some aerobic bacteria such as *Pseudomonas* or *Acinetobacter* were accounted for only 2.6 % relative abundance in the 42-strain multispecies community, although the *Pseudomonas* and *Acinetobacter* were strong biofilm former. Meanwhile, the facultative anaerobic bacteria *Citrobacter* and *Carnobacterium* dominated the biofilm (Visvalingam et al., 2019a). Therefore, the facultative anaerobic bacteria may have a competitive advantage against the strict aerobic bacterial groups during the development of the multispecies biofilm (Stewart and Franklin, 2008; Visvalingam et al., 2019a).

Moreover, Habimana et al. (2010) investigated a dual-species biofilm (72 h old) constructed by *Acinetobacter calcoaceticus* and STEC O157:H7, and STEC O157:H7 were covered by *A. calcoaceticus* in dual-species biofilm. It should be noticed that the deeper spatial distribution of

pathogenic anaerobic bacteria such as O157:H7 within a multispecies biofilm may help it survive the sanitation process and increase the food contamination risk (Habimana et al., 2010).

2.5.4.2. Bacteria Colonization Sequence

During beef fabrication, STEC carried by beef carcass could attach to food contact surfaces (e.g., conveyor belt) by cross-contamination (Wang, 2019). Meanwhile, it is likely other bacteria (spoilage, pathogens) present in the food processing facility are already forming multispecies biofilm (Wang, 2019). However, knowledge on the role of the bacteria present in the food processing facility is lacking.

Wang et al. (2015) investigated mixed biofilms of STEC serotypes O111:H8 and O157:H7. They noticed that the STEC serotype inoculated onto the surface first could become the dominant membership within mature biofilms (Wang et al., 2015). Therefore, the colonization sequence of the bacteria species could significantly influence the final microflora composition and predominant species within the multispecies biofilm (Wang, 2019). Meanwhile, the competitive advantage among the different STEC serotypes within the multispecies biofilm is also serotype-dependent (Wang, 2019). For example, STEC serotypes O26:H11 can dominate within mixed biofilms of O26:H11 and O157:H7 regardless of the inoculation sequence (Wang et al., 2012b). Hence, developing biofilms on food processing equipment with pre-selected non-pathogenic bacterial species may prevent STEC biofilm formation as a biocontrol strategy (Alegre et al., 2013).

2.5.4.3. Bacterial Culture Inoculum Concentration

Most previous research studying multispecies biofilm interaction applied different bacteria cultures at the same concentration (Wang, 2019). However, as indicated by Puga et al. in 2016, applying each bacteria species under equal concentration at the initial stage of multispecies biofilm development is not realistic because some species (e.g., *Pseudomonas* spp.) are commonly dominant in the food industry (Puga et al., 2016).

In the research by Pang et al. in 2018, *P. aeruginosa* had a better competitive advantage in the multispecies biofilm with lower *Salmonella* inoculation concentration (10^2 CFU/ml) compared with the same inoculation concentration (10^4 CFU/ml) (Pang and Yuk, 2018). It is believed the dominant *P. aeruginosa* (10^4 CFU/ml) could better occupy the surface and seize nutrients available for bacterial growth and biofilm development and further suppress the *Salmonella* (10^2 CFU/ml) growth (Pang and Yuk, 2018). Therefore, the initial inoculate concentration of different bacteria might influence the multispecies biofilm interaction (Pang and Yuk, 2018).

3. Chapter 3: Formation and transfer of multi-species biofilms containing *E. coli* O103:H2 on food contact surfaces to beef.

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3.1. Abstract

Interactions of Shiga-toxin producing *E. coli* (STEC; O103:H2) with lactic acid bacteria (LAB) or spoilage bacteria (SP) multispecies biofilms on polyurethane (TPU) and stainless steel (SS) were assessed at 10 °C and 25 °C under wet and dry conditions after 6, 30 & 60 d of storage. One LAB **T1**: *Carnobacterium piscicola* + *Lactobacillus bulgaricus,* and two SP **T2**: *Comamonas koreensis* + *Raoultella terrigena;* **T3**: *Pseudomonas aeruginosa* + *C. koreensis* were assessed for their ability to form multispecies biofilms with O103:H2. O103:H2 single-species biofilms served as a control positive (**T4**). Coupons were stored dry (20%-50% relative humidity; RH) or moist (60~90% RH) for up to 60 d, at which point O103:H2 transfer to beef and survival was evaluated. At 25 °C, **T3** decreased beef contamination with O103:H2 by 2.54 $\log_{10}$ CFU/g ($P < 0.001$). Overall, at 25 °C contamination of beef with O103:H2 decreased ($P < 0.001$) from 3.17 $\log_{10}$ CFU/g on d 6 to 0.62 $\log_{10}$ CFU/g on d 60. With 60 d dry biofilms on TPU, an antagonistic interaction was observed among O103:H2 and multispecies biofilm T1 and T3. *E. coli* O103:H2 was not recovered from T1 and T3 after 60 d but it was recovered (33%) from T2 and T4 dry biofilms. At 10 °C, contamination of beef with O103:H2 decreased ($P < 0.001$) from 1.38 $\log_{10}$ CFU/g after 6 d to 0.47 $\log_{10}$ CFU/g after 60 d. At 10 °C, recovery of O103:H2 from 60 d dry biofilms could only be detected after enrichment and was always higher for T2 than T4 biofilms. Regardless of temperature, the transfer of O103:H2 to beef from the biofilm on TPU was greater ($P < 0.001$) than SS. Moist biofilms also resulted in greater ($P < 0.001$) cell transfer to beef than dry biofilms at 10 and 25 °C. Development of SP or LAB multispecies biofilms with O103:H2 can either increase or diminish the likelihood of beef contamination. Environmental conditions such as humidity, contact surface type as well as biofilm aging all can influence the risk of beef being contaminated by STEC within multi-species biofilms attached to food contact surfaces.

3.2. Introduction

The prevalence of Shiga Toxigenic *E. coli* (STEC) in Canadian cattle was evaluated at two

western Canadian slaughter plants. In this research fecal samples (n=1794) were collected over two years from cattle trailers. Results showed that 94.4% of the fecal samples were positive for the serogroup O103 fallowed by O45 (93.1%), O26 (82.3%), O157 (78.8 %), O121 (66.1%), O111 (8.2%), and O145 (7.0%) (Stanford et al., 2016). Ruminants are considered the main reservoir of STEC. STEC can be transferred from hide and feces to the carcasses during processing (Bryan et al., 2015; PHAC, 2015). In 2019, 1462 STEC infections were reported to the Canadian National Enteric Surveillance Program (NESP) with approximately 73% of these caused by non-O157 (PHAC, 2020). Non-O157 serogroups causing disease in Canada have exceeded the number of O157-related since 2017 (PHAC, 2020). In 2019, non-O157 isolated from human infections were primarily represented by five serogroups: O26 (16%), O111 (10%), O103 (8%), O118 (3%), and O121 (3%) (PHAC, 2020).

Biofilm is defined as a community of microorganisms attached to a solid surface or to each other (Srey et al., 2013; Vogeleer et al., 2014; Adator et al., 2018). Bacterial cells within biofilms are embedded within a self-produced extracellular polymeric substance (EPS), which reduces their sensitivity to selective pressures such as heat, biocides, and antimicrobials (Srey et al., 2013; Vogeleer et al., 2014; Adator et al., 2018). Consequently, biofilms could contribute to the persistence of *E. coli* on the beef fabrication environment (Yang et al., 2018). Biofilms seems to be prominent within beef processing facilities, as more than 80% of generic *E. coli* isolated from beef fabrication equipment formed strong biofilms on stainless steel and were highly resistant to quaternary ammonium chloride (Yang et al., 2018). Therefore, STEC is likely to establish its presence on beef fabrication equipment through biofilm formation, even though biofilm-forming STEC is rare (Stanford et al., 2021). For example, research looking at STEC collected from live cattle showed that 93% (n=745) lacked biofilm-forming ability (Stanford et al., 2021). Despite only 3% of weak, 3% of intermediate, 1% of strong, and 0.3% of extreme biofilm-forming STEC among the 745 isolates from cattle, they may be related with HEP through surviving the sanitation process and persistent in the beef fabrication facility (Stanford et al., 2021). Interestingly, multiple previous studies demonstrated that non-pathogenic bacteria originating from beef facilities

(*Comamonas testosterone* or/ *Acinetobacter calcoaceticus*) can enhance STEC O157:H7 biofilm formation (Marouani-Gadri et al., 2009; Habimana et al., 2010).

STEC biofilms on contact surfaces have generally been investigated using single-species biofilms (Wang et al., 2012a; Adator et al., 2018; Yang et al., 2018). However, biofilms that form within beef processing facilities are typically composed of multiple species (Wang et al., 2018; Visvalingam et al., 2019a; Visvalingam et al., 2019b). For example, both lactic acid bacteria (LAB) (e.g., *Carnobacterium* spp.) and spoilage bacteria (e.g., *Raoultella* spp., *Pseudomonas* spp.) were isolated from conveyor belt biofilms within a beef processing facility (Wang et al., 2018). Functional characteristics of bacteria within multispecies biofilms can substantially differ from that exhibited within single-species biofilms (Burmolle et al., 2014; Pang and Yuk, 2018). Thus, to enhanced risk assessment and improve pathogen intervention strategies, it is important to investigate the interactions of STEC with other bacterial species within biofilms (Giaouris et al., 2015). The objectives of this study were to 1) evaluate potential synergistic and antagonistic interactions of STEC (O103:H2) with either LAB or spoilage bacteria (SP) within multispecies biofilms formed on thermoplastic polyurethane (TPU) or stainless steel (SS); 2) determine the extent of transfer of O103:H2 cells from single and multispecies biofilms to beef at different storage times, temperatures, and humidity and 3) determine the capacity of STEC to survive within single vs multispecies biofilms.

3.3. Material and Methods

3.3.1. Bacteria Strains and Culture Conditions

Nine STEC strains including 7 serogroups, 12 SP, and 12 LAB were assessed for their suitability to use in this study (Table 3-1). STEC strains were cultured on MacConkey agar plates (Hardy Diagnostics Inc., Santa Maria, USA), while SP and LAB bacteria were cultured on Trypticase Soy Agar (TSA; Becton, Dickinson and Company, MD, USA) at 25 ℃. A single colony of each STEC/ LAB/ and SP strain was transferred from each plate into individual 10 mL Lysogeny Broth no salt (LB-NS; Tryptone 10 g/L and yeast extract 5 g/L) and grown to a cell density of 10^8

CFU/mL. The incubation time required for the strains to reach the early stationary phase varied from 24 to 72 h (Visvalingam et al., 2019a). Cultures were subsequently diluted to 10^6 CFU/mL for use in biofilm formation assessment assays.

To simulate the nutrient profile within beef fabrication plants, LB-NS broth was supplemented with sterile beef purge (Pang and Yuk, 2018) that originated from a different lot of a vacuum-packed beef product (i.e., the eye of round) for all replications. The vacuum package was opened, the beef purge was collected and distilled water was added at a ratio of 1:6. The aqueous solution was then passed through a 0.45 μm sterile filter (Midelet and Carpentier, 2002), protein content was determined using a Bradford kit (Thermo Scientific, Rockford, IL) and the filtrate was stored at -20 °C. The filtrate was mixed with LB-NS broth (10% v/v; mLB-NS) for use in biofilm formation assays.

Table 3-1. STEC, LAB, and spoilage bacteria were selected to perform the biofilm-forming ability test.

Serotype	Strain ID	Source	Category
O26: H11	00-3941	Human	STEC
O45: H7	05-6545	Human	STEC
O103: H2	99-2076	Human	STEC
O111: NM	CFS3	Human	STEC
O121: H19	03-2832	Human	STEC
O145: H2	75-83	Human	STEC
O157: H7	1934	Beef	STEC
O157: H7	1931	Hamburger	STEC
O157: H7	R508	Bovine/feces	STEC
Lactobacillus sakei *	S19	Vacuum-packaged meat	LAB
Leuconostoc gelidum *	S21	Vacuum-packaged meat	LAB
Carnobacterium divergens *	B1	Vacuum-packaged meat	LAB
Carnobacterium maltaromaticum *	LAB9_67	Meatpacking plant	LAB
Pediococcus acidilactici	ATCC 8081	Fermented milk	LAB
Leuconostoc mesenteroides	A5	Meat	LAB
Lactobacillus bulgaricus	ATCC11842	Yogurt	LAB
Lactobacillus curvatus	133L	Meat starter culture	LAB
Lactobacillus sakei	LB 808 (S206)	Unknown	LAB
Carnobacterium piscicola	M5L1	Vacuum package pork	LAB
Carnobacterium divergens	ATCC 35677	Vacuum package minced beef	LAB
Aerococcus viridans	ATCC 11563	Air sample	LAB
Generic *E. coli* *	8_77	Meatpacking plant	Spoilage
Generic *E. coli* *	7_16	Meatpacking plant	Spoilage
Hafnia alvei *	S1	Vacuum-packaged meat	Spoilage

Rahnella sp. *	S8	Vacuum-packaged meat	Spoilage
Serratia sp. *	S10	Vacuum-packaged meat	Spoilage
Sphingopyxis sp. *	03_68	Meatpacking plant	Spoilage
Comamonas koreensis *	25_64	Meatpacking plant	Spoilage
Raoultella terrigena *	ENT25_16	Meatpacking plant	Spoilage
Yersinia enterocolitica	UN2814 602	Unknown	Spoilage
Pseudomonas aeruginosa	ATCC 7700	Well water	Spoilage
Listeria monocytogenes	GLM1	Meat processing plant	Spoilage
Listeria monocytogenes	GLM3	Meat processing plant	Spoilage

Asterisk indicated the strain was isolated by Agriculture and Agri-Food Canada, (Wang et al., 2018).

Non-asterisk indicated the strain was collected from the University of Manitoba.

3.3.2. Biofilm Forming Ability Determination

3.3.2.1. Crystal Violet Method Assessing Biofilm Formation

To assess biofilm formation, fresh cultures of each strain were diluted in mLB-NS to 10^6 CFU/mL. Then, 200 µL of the 10^6 CFU/mL culture was transferred to designated wells in a 96-well microplate as described by Wang et al. (2018). Microplates were subsequently incubated at either 10 or 25 °C for 6 d. At this point, microplates were washed three times with 300 µL of Butterfield's Phosphate Buffer (BPB) per well using a microplate washer (405 LS, BioTek®, Winooski, USA,). Washed plates were air-dried for 30 min in a biosafety level 2 cabinet (BSL2), and 200 µL of methanol was transferred to each well. After 15 min, the methanol was aspirated and 200 µL of 0.1% crystal violet (CV) was added to each well (Wang et al., 2016a). After 15 min, the microplate was washed three times with 300 µL BPB per well, and the residual crystal violate in each well was solubilized in 200 µL of 85% ethanol (Wang et al., 2012a). Biofilm-forming ability was determined indirectly by measuring residual chromophore using a microplate reader at 630 nm (BioTek ELx800; BioTek Instruments Inc., Winooski, USA). Three repetitions were

performed for each isolate (n=16 x 3); with a total of 48 wells per isolate. Each isolate was categorized according to its biofilm-forming ability, with three microplate wells containing mLB-LS only serving as negative controls. The positive controls included *E. coli* O157:H7 R5O8, a known strong biofilm former (Adator et al., 2018).

To classify biofilm-forming ability, optical density cut-offs (ODc) were calculated as three standard deviations from the mean value of the control negative as described by Adator et al. 2018. Classifications included OD ≤ ODc = non biofilm former; ODc < OD ≤ 2ODc = weak biofilm former; 2ODc < OD ≤ 4ODc = intermediate biofilm former; 4ODc < OD = strong biofilm former. The intermediate/ strong biofilm formers identified at either 10 or 25 ℃ were selected to form multispecies biofilms subsequent experiments.

3.3.2.2. STEC Curli and Cellulose Expression

STEC strains that possess curli fimbriae and produce cellulose have been shown to be strong biofilm formers (Adator et al., 2018). To assess curli, fresh overnight cultures were plated onto Congo red agar (10 g/L casamino acids, 1 g/L yeast extract, and 20 g/L agar), supplemented with 20 µg/mL Coomassie brilliant blue dye (Sigma Aldrich, St. Louis, USA) and 40 µg/mL Congo red dye (Sigma Aldrich), (CRI) (Adator et al., 2018). Cellulose production was assessed using fresh overnight cultures plated onto LB agar (Hardy Diagnostics CulGenex™, Santa Maria, USA) supplemented with 200 mg/L Calcofluor dye (Sigma Aldrich) (Wang et al., 2013b). Plates were incubated at 28 ℃ for 72 h and cellulose production was assessed by measuring fluorescence at 366-nm (Wang et al., 2013b). Duplicate samples were included in each experiment, with experiments replicated three times. Curli and cellulose production were defined as previously described (Gaylen et al., 2006; Wang et al., 2013b) as follows:

(A) cellulose negative – no colony fluorescence at 366-nm; (B) cellulose positive – colony fluorescence at 366-nm; (C) curli negative – smooth and white colony; (D) curli positive – red, dry, and rough/ brown, dry, and rough colonies (Figure 8-1).

3.3.3. Multispecies Biofilm Assays

3.3.3.1. STEC O103:H2 Multispecies Biofilm

Based on results obtained from the crystal violet assays, strong and intermediate biofilm formers were selected from STEC, LAB and SP isolate for use in multispecies biofilm experiments (Figure 3-1). Four LAB: *Lactobacillus bulgaricus* (strong), *Lactobacillus curvatus* (strong), *Carnobacterium divergens* B1 (intermediate) and *Carnobacterium piscicola* (intermediate), and one spoilage bacterium: *Pseudomonas aeruginosa* (strong) were selected based on their ability to form strong/ intermediate biofilms at 25 °C (Figure 3-1A). Additionally, one LAB: *Lactobacillus sakei* S19 (intermediate) and three spoilage bacteria: *Serratia* sp. (strong), *Comamonas koreensis* (weak), and *Raoultella terrigena* (strong) were also selected based on their biofilm-forming ability at 10 °C (Figure 3-1B). Regarding STEC strain selection, none of the tested STEC strains met the criteria as strong/intermediate biofilm formers at 10 °C, but several were strong biofilm formers at 25 °C. Of these, O103:H2 (*stx*1 positive) was selected due to its high prevalence in fecal samples obtained from Canadian cattle before slaughter (Stanford et al. 2016).

LAB and SP bacterial mixed-biofilms were formed first and subsequently, O103:H2 was introduced into the mixed-species biofilm as described by Wang et al. (2013). Briefly, fresh cultures of each LAB and SP strain were diluted in mLB-NS to 10^6 CFU/mL and then mixed according to the factorial arrangements shown in Table 3-2. Around 200 µL spoilage or LAB cultures (10^6 CFU/mL) were aliquoted into microplate wells, with two sets of microplates for each experiment. Mature biofilms were allowed to form in the plates at 10 and 25 ℃ over 6 d. After 6 d, the supernatant in each well was aspirated, and each well was washed with 200 µL BPB to remove free and loosely attached cells. At this point, 200 µL fresh O103:H2 culture (10^3 CFU/mL) in mLB-NS was aliquoted into designated wells. Microplates were incubated for an additional 6 d and thereafter washed three times with 300 µL BPB. One plate was used for enumeration and the other was used to assess the persistence of O103:H2 in mixed biofilms. One column in the microplate was retained as a positive control with O103:H2 only, and a second column served as a negative control and received no inoculant. Each column (8 wells/ column) was regarded as one

observation, and the experiment was repeated three times in duplicate for each strain combination.

Table 3-2. Factorial design of strains combination.

	Lactic Acid Bacteria Combination				
	L. sakei S19	*C. divergens* B1	*L. bulgaricus* ATCC11842	*L. curvatus* 133L	*C. piscicola* M5L1
L. sakei S19					
C. divergens B1	+				
L. bulgaricus ATCC11842	+	+			
L. curvatus 133L	+	+	+		
C. piscicola M5L1	+	+	+	+	
	Spoilage Bacteria Combination				
	Serratia sp. S10	*R. terrigena* ENT25_16	*C. koreensis* 25_64	*P. aeruginosa* ATCC7700	
Serratia sp. S10					
R. terrigena ENT25_16	+				
C. koreensis 25_64	+	+			
P. aeruginosa ATCC7700	+	+	+		

3.3.3.2. Biofilm STEC Enumeration

STEC enumeration was performed immediately after the microplate was washed. Buffered peptone water (200 µL; BPW, Hardy Diagnostics Inc.) was dispensed into each corresponding well and a sterile pipette tip was used to detach the biofilm by scraping the wall and bottom of each well (Wang et al., 2013b). Subsequently, microplates were sonicated at 40 kHz (Branson 2800, Branson Ultrasonics Co., Danbury, USA) for 1 min (Uhlich et al., 2006) and equal volumes of BPW from each well were pooled to generate 1 mL of culture for 10-fold dilution (Wang et al., 2013b). O103:H2 was enumerated on MacConkey agar overlaid with TSA using the drop plate

method (Herigstad et al., 2001). For the drop plate method, five drops (10 µL/drop) were dispensed on each plate, which was then incubated for 24 h at 37 °C. Recovered colonies were confirmed as *E. coli* O103 via agglutination (SSI Diagnostica, Hillerød, Denmark) and PCR (Debroy et al., 2011).

3.3.3.3. Biofilm STEC Persistence and Survival

The second set of microplates containing multispecies biofilms was used to assess the survival of O103:H2 after desiccation. Microplates were maintained at 10 or 25 °C for one month at ~20-30% relative humidity (RH). Then, modified tryptone soy broth (200 µL; mTSB; Oxoid Ltd., Nepean, Canada) was added to each well, and plates were incubated for 24 h at 37 °C. A 3 µl aliquot of mTSB was removed from each well, spotted onto MacConkey agar, and verified as *E. coli* as described above.

3.3.4. Multispecies Biofilm Formation on Food Contact Surfaces and STEC Transfer to Beef

3.3.4.1. Bacteria Strain and Culture Combination

Based on the O103:H2 cell numbers (Figure 3-2) and recovery rate (Table 3-4) from multispecies biofilms (n = 16), three species combinations were selected to form multispecies biofilm on food contact surfaces. These included **T1**: *C. piscicola* + *L. bulgaricus*; two SP combinations **T2**: *C. koreensis* + *R. terrigena* and **T3**: *P. aeruginosa* + *C. koreensis*. Biofilms were formed on thermoplastic polyurethane (TPU) and 304 stainless-steel (SS), common components of conveyor belts and food processing surfaces, respectively.

3.3.4.2. Polyurethane and Stainless-steel Coupons Preparation

Thermoplastic polyurethane coupons were prepared by sectioning a 2-ply white urethane smooth top surface food grade conveyor belt (2E8U 0/02 White, NuTech Conveyor Components, Milton, CA) into 2 cm × 2 cm pieces. Sanitized by soaking overnight in hydrogen peroxide (Accel®

PREVention$^{TM/MC}$, Diversey^{TM}, Fort Mill, USA). Coupons were then washed and soaked in sterile distilled water for 1 h.

Stainless steel 304 coupons (2 cm × 2 cm; Pegen Industries Inc., Stittsville, CA) were washed in distilled water and placed in an ultrasonic water bath for 20 min at 60℃ (Adator et al., 2018). Coupons were further sonicated in 15% phosphoric acid for 20 min at 60℃, an additional 20 min in distilled water, before dry-sterilization in an autoclave.

3.3.4.3. Dry and Wet Multispecies Biofilm Formation

Coupons were transferred to sterile Petri dishes (60 x 15 mm; VWR^{TM}, Radnor, USA) and either the spoilage or LAB mixed bacterial cultures (10^6 CFU/mL) were added to each coupon (5 mL). Coupons were placed at either 10 or 25 ℃, for 6 d to form mature biofilms. After 6 d, coupons were washed with BPB three times (10 mL/ coupon) and placed in a new sterile Petri dish. Aliquots (5 mL) of O103:H2 culture (10^3 CFU/mL) were then added to the preformed biofilms and incubated for an additional 6 d at each assigned temperature. Positive and negative controls were included as described above.

Coupons were stored under dry (20~50% RH) or moist conditions (60~90% RH), with moist biofilms being sprayed with sterile water (150 µl/ coupon) daily. A subset of the TPU and SS coupons were used to determine the extent to which O103:H2 was transferred to beef. The second set of coupons was used for STEC enumeration from biofilms after 6, 30, and 60 d of storage.

3.3.4.4. Beef Samples Preparation to Test O103:H2 Transfer

Retail whole cuts eye of round beef with the fat cap were purchased in a local grocery store and kept at 4 °C before use. A 5% lactic acid solution was used to wash the meat surface to reduce background flora and pieces were subsequently immersed in lactic acid for 1 min (Youssef et al., 2013). Beef pieces were allowed to drain and were cut into 3 cm x 3 cm pieces using an aseptic technique and stored at 4 °C for up to 24 h.

3.3.4.5. STEC Transfer from Biofilms to Beef and STEC Biofilm Enumeration

For STEC transfer, beef pieces were placed on TPU or SS coupons, and a 50 g weight was placed on top of each piece to exert a pressure of 7.35 kPa (Flores et al., 2006). A piece of wax paper was placed between the weight and the meat to avoid direct contact with the weight during the 5 min contact time. Beef pieces were then removed from each coupon and placed in a Whirl-Pak bag (Nasco®; Madison, USA) along with 9-mL of BPW to obtain a ten-fold dilution and homogenized using a stomacher (Intersciences Inc., Markham, Canada) for 1 min. For STEC enumeration, 10-fold dilutions were prepared and plated on TSA overlayed MacConkey agar (Wu, 2008). Plates were incubated for 24 h at 37 °C and isolates were confirmed as described above.

To quantify STEC on TPU and SS, coupons were placed in whirl-Pak bags along with 9-mL of BPW to obtain ten-fold dilution and sonicated for 1 min (Marouani-Gadri et al., 2009). For enumeration, the drop plate method was used as outlined above. Samples on MacConkey plates (undetectable levels) that did not produce colonies after 24 h at 37°C, were subject to enrichment in mTSB for 24 h at 37°C, before spread plating on MacConkey agar.

3.3.5. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed at the Manitoba Institute for Materials (MIM) to visualize dry biofilm formation on SS and TPU surfaces as described previously (Adator et al., 2018). The TPU and SS coupons were fixed (neutral buffered 10% formalin solution, Sigma Aldrich) for 2 h and then washed with BPB for 30 min. The coupons were dried for 4 hours at room temperature in a BSL2 cabinet, and the TPU surface was Gold-Palladium-coated (Denton Vacuum Desk II, Moorestown, US) in the high-vacuum mode on the following day. Biofilm structures were observed using a QuantaTM 650 FEG scanning electron microscope (FEI CO., Hillsboro, US) in the high-vacuum mode at 5 KV.

3.3.6. Statistics Analysis

All experiments were performed three times. The Proc Mixed procedure of the Statistical Analysis System (Cary, USA) was used to analyze the data with the least mean separation accomplished using the PDIFF option. For biofilm-forming ability and multispecies biofilm microplate assays, a factorial model was applied to analyze the main effects of bacterial strain, temperature, and their two-way interaction. For beef contaminated by O103:H2 on food contact surfaces, effects of species, contact surface, storage time, and humidity along with the appropriate interactions were tested. For all statistical analyses, significance was declared at $P \leq 0.05$.

3.4. Results

3.4.1. Biofilm Formation Abilities using the crystal violet assay method and Strain Selection

Isolates varied substantially in their biofilm-forming ability (Figure 3-1). A bacterial isolate by temperature interaction was identified ($P < 0.001$) as isolates more readily formed biofilms at 25 °C than others. For example, at 25 °C several strains showed strong biofilm-forming abilities, which included all the STEC strains except O145:H2 (weak) and O157:H7 1934 (intermediate), (Figure 3-1A). Within the LAB, *L. bulgaricus*, *L. curvatus*, *Lactobacillus sakei* S206, and *Aerococcus viridans* were all classified as strong biofilm formers at 25 °C. Likewise, *P. aeruginosa, Rahnella* sp.*, R. terrigena,* and *E. coli* (8_77) also formed strong biofilms at this temperature. In contrast, *Serratia* sp. and *R. terrigena* isolates were able to form strong biofilms at 10 °C, while other isolates were intermediate or weak biofilm formers at this temperature (Figure 3-1B).

Evaluation of curli and cellulose indicated that strain O26: H11 (00-3941), O103: H2 (99-2076), O111: NM (CFS3), O121: H19 (03-2832), O157: H7 (R508), and *E. coli* (8_77) showed both curli and cellulose producing ability at 25 °C (Table 3-3). Based on these data, *L. sakei* S19, *Serratia* sp., *C. koreensis*, *R. terrigena*, *L. bulgaricus*, *L. curvatus*, *C. divergens* B1, *C. piscicola*, *P. aeruginosa,* and O103:H2 were selected to further investigate multi-species biofilms.

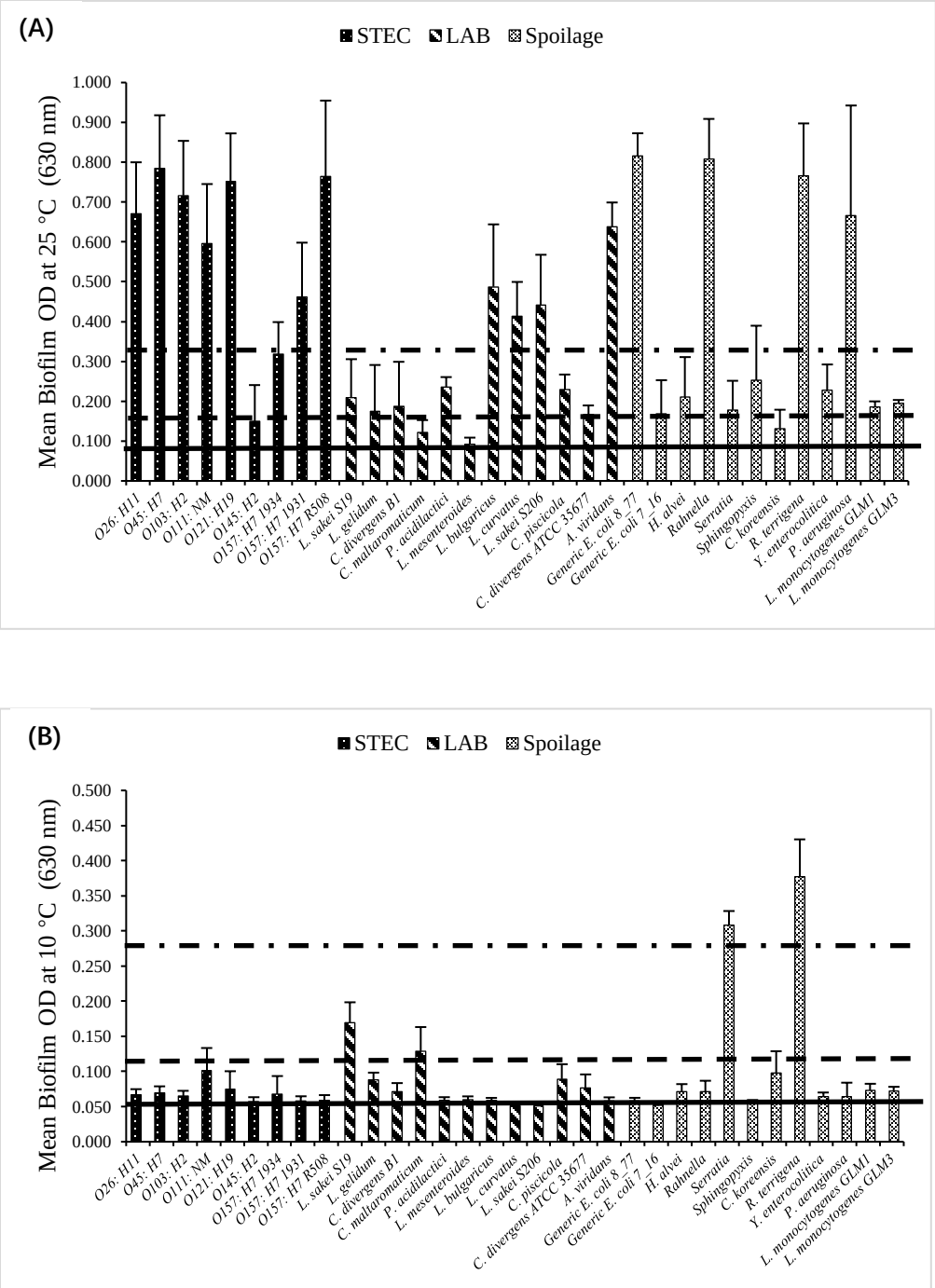

Figure 3-1. STEC biofilm formation on microplates after 6 days at (A) 25 °C and (B) 10 °C. Biofilms formed by each strain were determined in 3 replicate experiments. Horizontal lines going from bottom towards the top are non (OD < ODc), weak (ODc < OD < 2ODc), intermediate (2ODc < OD < 4ODc) and strong (4ODc < OD) biofilm formers. The biofilm-forming ability differed ($P < 0.001$) with incubation temperature among strains.

Table 3-3. Curli and cellulose production of the STEC and generic *E. coli* strains with different biofilm-forming abilities at 25 °C.

Strain	Cellulose	Curli	Biofilm forming ability
O26: H11 (00-3941)	+	+	Strong
O45: H7 (05-6545)	+	-	Strong
O103: H2 (99-2076)	+	+	Strong
O111: NM (CFS3)	+	+	Strong
O121: H19 (03-2832)	+	+	Strong
O145: H2 (75-83)	-	-	Weak
O157: H7 (1934)	-	-	Intermediate
O157: H7 (1931)	-	-	Strong
O157: H7 (R508)	+	+	Strong
Generic *E. coli* (8_77)	+	+	Strong
Generic *E. coli* (7_16)	-	-	Intermediate

3.4.2. *In-vitro* Multispecies Biofilms and STEC Interaction

3.4.2.1. STEC Enumeration from *In-vitro* Multispecies Biofilms

Overall, O103:H2 numbers within the multispecies biofilm vary ($P < 0.001$) by strain combination (Figure 3-2). None of the tested LAB bacteria altered O103:H2 counts ($P > 0.05$) when compared with the positive control (5.10 $\log_{10}$ CFU/mL) and the numbers of O103:H2 recovered from LAB biofilms ranged from 4.76 – 5.13 $\log_{10}$ CFU/mL. When O103:H2 was exposed to all of the SP biofilms, colonization by O103:H2 was reduced ($P < 0.05$), with the highest reduction (2.23 $\log_{10}$ CFU/mL) ($P < 0.001$) occurring with mixed *P. aeruginosa* and *C. koreensis* biofilms.

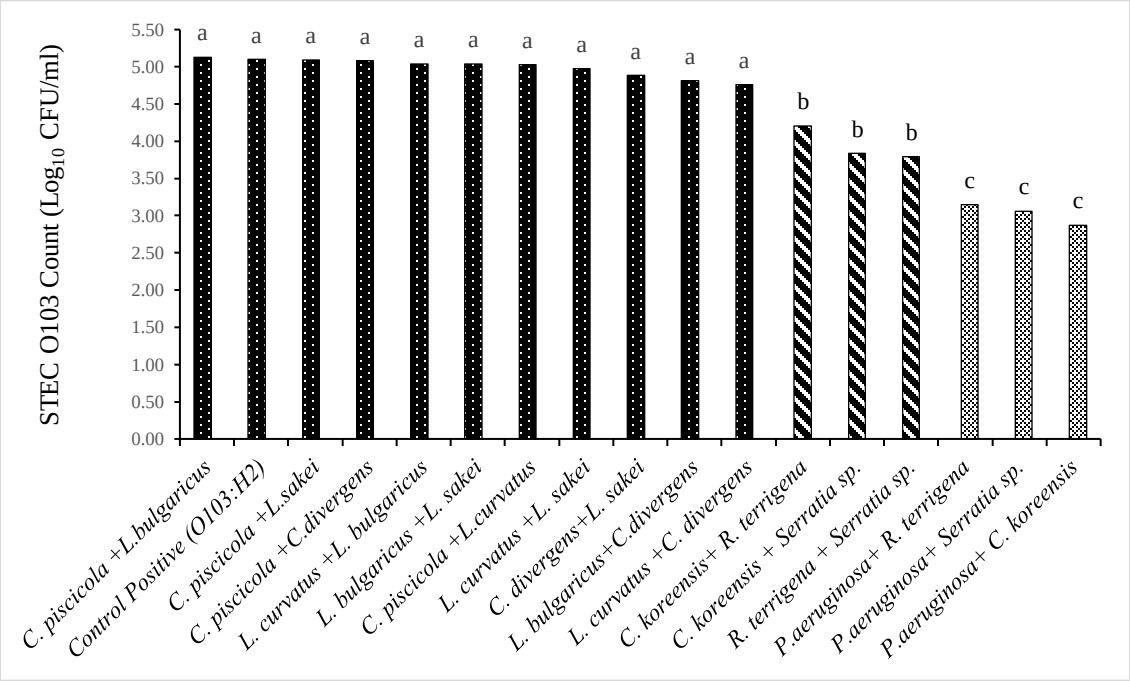

Figure 3-2. Least squares mean of STEC O103:H2 enumerated from 6-d old moist multispecies biofilms formed in microplates (SEM = 0.17). a,b,c: Least squares means with different superscript letter differ ($P < 0.05$).

3.4.2.2. STEC Survival within 30-day Old Dry Multispecies Biofilms

After biofilms were keep dry for 30 d. *Escherichia coli* O103:H2 was not recovered from the following biofilms combinations *P. aeruginosa* + *C. koreensis* and *P. aeruginosa* + *Serratia* sp. kept at 25 °C (Table 3-4). Interestingly, the biofilm combination of *P. aeruginosa* + *R. terrigena* did not alter the recovery of O103:H2 (33%) as compared to O103:H2 single species biofilms (33%). Regarding multispecies biofilms composed by LAB species, O103:H2 was recovered from all LAB biofilms (100%); interestingly, O103:H7 recovery from controls (O103:H2 single specie biofilms) was lower (33%) (Table 3-4). Similarly, O103:H2 was 100% recovered from mixed biofilms containing *C. koreensis* + *R. terrigena*.

In contrast, at 10 ℃, O103:H2 recovery from all mixed-species biofilms were much lower (0 – 50%) than at 25 ℃ (Table 3-4). Interestingly, when looking at controls, *E. coli* O103 was better able to survive within biofilms formed and kept at 10 ℃ than from within those formed and kept at 25 ℃ (50 % survival vs 33.33% survival). However, some multispecies biofilms were able to

reduce O103 recovery to 0 %, including *R. terrigena* +*Serratia sp.*, *P. aeruginosa* + *C. koreensis*, and *C. divergens* B1+*L. sakei* S19. While other combinations including *C. koreensis* +*Serratia* sp., *P. aeruginosa* +*Serratia* sp., and *C. koreensis* + *R. terrigena* showed not effect on survival (Table 3-4).

Table 3-4. Recovery of STEC O103:H2 after 24 h of enrichment from multispecies dry biofilms stored for 30 d at 10 and 25 °C.

Strain combination	Recover from 10 °C, % (n/N)	Recover from 25 °C, % (n/N)
R. terrigena +*Serratia* sp.	0.00 (0/6)	66.67 (4/6)
C. koreensis +*Serratia* sp.	50.00 (3/6)	83.33 (5/6)
P. aeruginosa +*Serratia* sp.	50.00 (3/6)	0.00 (0/6)
C. koreensis + *R. terrigena*	50.00 (3/6)	100.00 (6/6)
P. aeruginosa + *R. terrigena*	33.33 (2/6)	33.33 (2/6)
P. aeruginosa + *C. koreensis*	0.00 (0/6)	0.00 (0/6)
C. divergens B1+*L. sakei* S19	0.00 (0/6)	100.00 (6/6)
L. bulgaricus +*L. sakei* S19	16.67 (1/6)	100.00 (6/6)
L. curvatus +*L. sakei* S19	0.00 (0/6)	100.00 (6/6)
C. piscicola +*L.sakei* S19	16.67 (1/6)	100.00 (6/6)
L. bulgaricus +*C.divergens* B1	16.67 (1/6)	100.00 (6/6)
L. curvatus +*C. divergens* B1	16.67 (1/6)	100.00 (6/6)
C. piscicola +*C.divergens B1*	16.67 (1/6)	100.00 (6/6)
L. curvatus +*L. bulgaricus*	33.33 (2/6)	100.00 (6/6)
C. piscicola +*L.bulgaricus*	33.33 (2/6)	100.00 (6/6)
C. piscicola +*L.curvatus*	0.00 (0/6)	100.00 (6/6)
Control positive (O103:H2)	50.00 (3/6)	33.33 (2/6)

3.4.3. Multispecies Biofilm Formation on Food Contact Surfaces and STEC Transfer to Beef

At 25 °C, *P. aeruginosa* + *C. koreensis* (T3) biofilms reduced ($P < 0.001$) the transfer of O103:H2 to beef by 2.54 $\log_{10}$ CFU/g (Figure 3-3. A, B, C). Mixed species *C. piscicola* + *L. bulgaricus* (T1) and *C. koreensis* + *R. terrigena* (T2) biofilms did not alter ($P > 0.05$) the transfer of O103:H2 cells to beef. Overall transfer of O103:H2 to beef from biofilms formed on TPU (2.14 $\log_{10}$ CFU/g) was greater ($P < 0.001$) than that from SS (1.40 $\log_{10}$ CFU/g). Transfer of O103:H2 to beef decreased ($P < 0.001$) with biofilm aging, from 3.17 log on d 6 to 1.52 $\log_{10}$ CFU/g on d 30 and 0.62 $\log_{10}$ CFU/g on d 60. Reductions in the transfer of O103:H2 to beef were highest for 6 d T3 mixed biofilms grown on TPU. Overall moist biofilms transferred more O103:H2 to beef (2.93 $\log_{10}$ CFU/g) than dry biofilms (0.61 $\log_{10}$ CFU/g) regardless the surface type ($P < 0.001$).

At 10 °C, none of the multispecies biofilms reduced or enhanced the transfer of O103:H2 to beef as compared to the positive control ($P > 0.05$) (Figure 3-3. D, E, F). Transfer of O103:H2 to beef was also higher ($P < 0.001$) from TPU (1.14 $\log_{10}$ CFU/g) than from SS surfaces (0.55 $\log_{10}$ CFU/g). Results also showed that moist biofilms were more likely to contaminate beef (1.58 $\log_{10}$ CFU/g) than dry biofilms (0.10 $\log_{10}$ CFU/g) ($P < 0.001$). At 10 °C, contamination of beef with O103:H2 decreased as biofilms aged, from 1.38 $\log_{10}$ CFU/g after 6 d to 0.47 $\log_{10}$ CFU/g after 60 d. ($P < 0.001$).

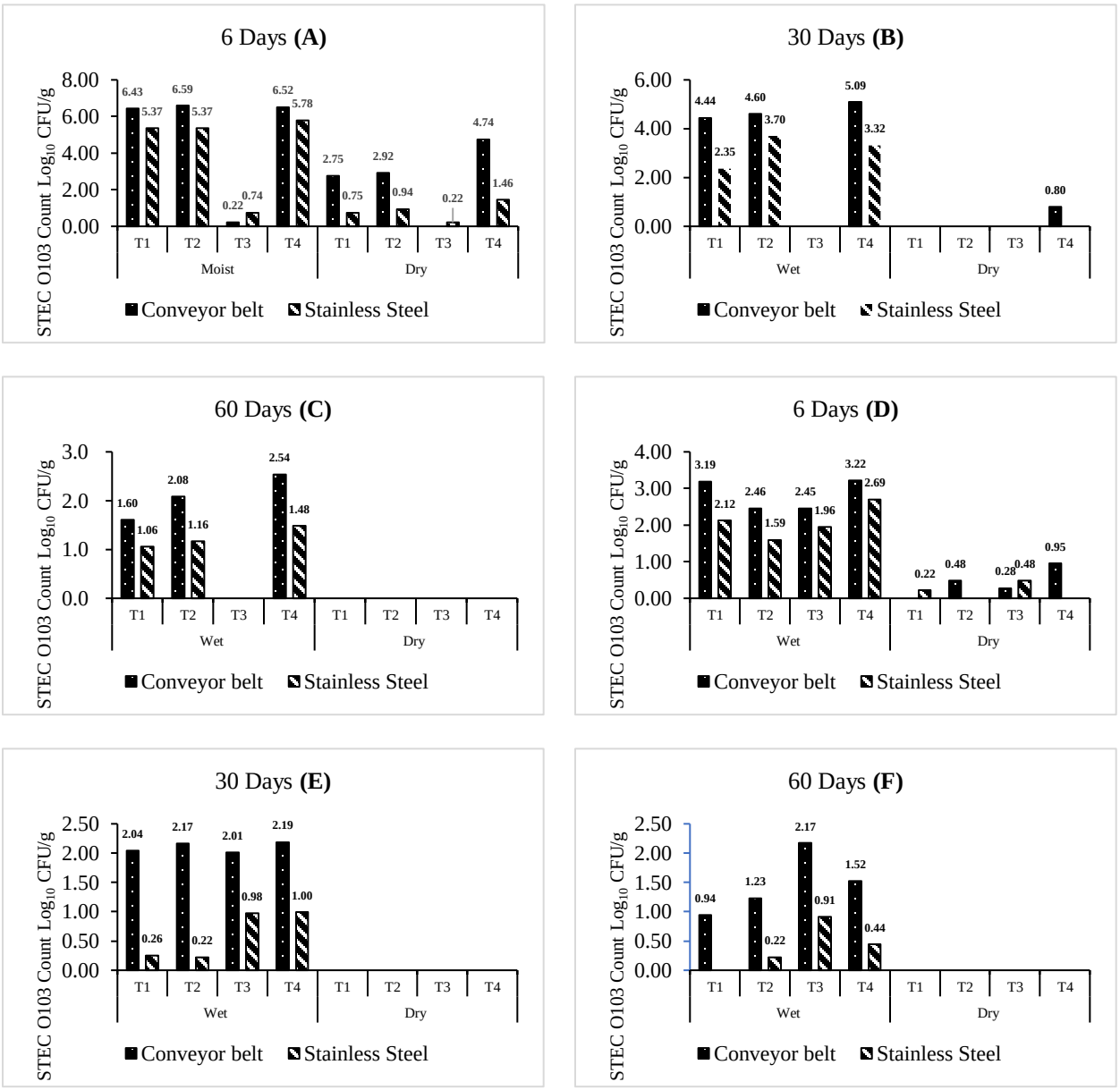

Figure 3-3. A, B, C; *E. coli* O103:H2 cells (CFU/g) transferred to beef from moist or dry multispecies biofilms formed at 25 °C for 6, 30, and 60 days. D, E, F; *E. coli* O103:H2 cells (CFU/g) transferred to beef from moist or dry multispecies biofilms formed at 10 °C for 6, 30, and 60 days. The four-strain combination were T1) *C. piscicola* + *L. bulgaricus*; T2) *C. koreensis* + *R. terrigena*; T3) *P. aeruginosa* + *C. koreensis*; and T4) STEC O103:H2 Control.

3.4.4. O103:H2 Recovery from Dry Biofilms

At 25 °C, no O103:H2 was recovered (0%) from 60 d dry biofilms on SS, even after enrichment (Table 3-5). On TPU, O103:H2 was recovered from approximately 33% of dry 60 d

multispecies of *C. koreensis* + *R. terrigena* (T2) and control positive (T4) biofilms. *E. coli* O103:H2 was not recovered from the following multispecies biofilms combinations *C. piscicola* + *L. bulgaricus* (T1) and *P. aeruginosa* + *C. koreensis* (T3).

At 10 °C after enrichment, O103:H2 was most often recovered from mixed biofilms of T2 formed on TPU/SS and stored for 60-d (Table 3-5). Recoveries of O103:H2 from dry biofilms on TPU ranked as T2 (89%) > T4 (44%) > T1 (33%) > T3 (22%). The highest O103:H2 recovery rate from dry biofilm on SS surface being T2 (33%) > T4 (11%) = T1 (11%) > T3 (0%).

Table 3-5. Recovery of STEC O103:H2, with and without enrichment from dry multispecies biofilms stored at 25 °C or 10 °C for 60 days.

Surface	Strain combination	Recover without enrichment, % (n/N)	Recover with enrichment, % (n/N)	Total recover, % (n/N)
25 °C				
TPU	T1: *C. piscicola* +*L. bulgaricus*	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	T2: *C. koreensis* + *R. terrigena*	0.00 (0/9)	33.33 (3/9)	**33.33 (3/9)**
	T3: *P. aeruginosa* + *C. koreensis*	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	T4: Control positive (O103:H2)	0.00 (0/9)	33.33 (3/9)	**33.33 (3/9)**
SS	T1: *C. piscicola* +*L .bulgaricus*	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	T2: *C. koreensis* + *R. terrigena*	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	T3: *P. aeruginosa* + *C. koreensis*	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	T4: Control positive (O103:H2)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
10°C				
TPU	T1: *C. piscicola* +*L. bulgaricus*	0.00 (0/9)	33.33 (3/9)	33.33 (3/9)
	T2: *C. koreensis* + *R. terrigena*	11.11 (1/9)	87.50 (7/8)	**88.89 (8/9)**
	T3: *P. aeruginosa* + *C. koreensis*	0.00 (0/9)	22.22 (2/9)	22.22 (2/9)
	T4: Control positive (O103:H2)	0.00 (0/9)	44.44 (4/9)	44.44 (4/9)
SS	T1: *C. piscicola* +*L. bulgaricus*	0.00 (0/9)	11.11 (1/9)	11.11 (1/9)
	T2: *C. koreensis* + *R. terrigena*	0.00 (0/9)	33.33 (3/9)	**33.33 (3/9)**
	T3: *P. aeruginosa* + *C. koreensis*	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	T4: Control positive (O103:H2)	0.00 (0/9)	11.11 (1/9)	11.11 (1/9)

3.4.5. Scanning Electron Microscopy

After 60 d storage, dry multispecies biofilm composed of *C. koreensis* + *R. terrigena* (T2)

was observed as a multilayer structure with rod shape bacteria covered by extensive EPS at both 25 °C (Figure 3-4) and 10 °C (Figure 3-5). Interestingly, control positive (T4) dry biofilm was displayed differently after 60 d storage at 25 °C (Figure 3-4) and 10 °C (Figure 3-5). At 25 °C, a well-developed multilayer T4 biofilm extensively covers the whole TPU surface. However, no individual bacteria and/or EPS were observed when the T4 biofilm was stored at 10 °C for 60 d.

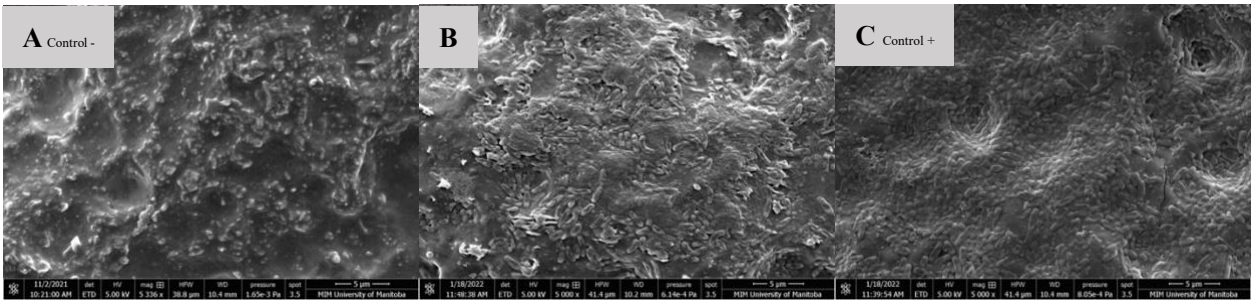

Figure 3-4. SEM of A) TPU coupon used as control negative, no bacteria observed. B) T2: *C. koreensis* + *R. terrigena*; and C) T4: STEC O103:H2 Control positive 25 °C dry biofilms at day 60 on a TPU surface.
In panels **B**) and **C**) a well-developed multilayered biofilm is displayed; the rod-shaped bacteria are dominant in biofilm and covered within the extensive EPS matrix.

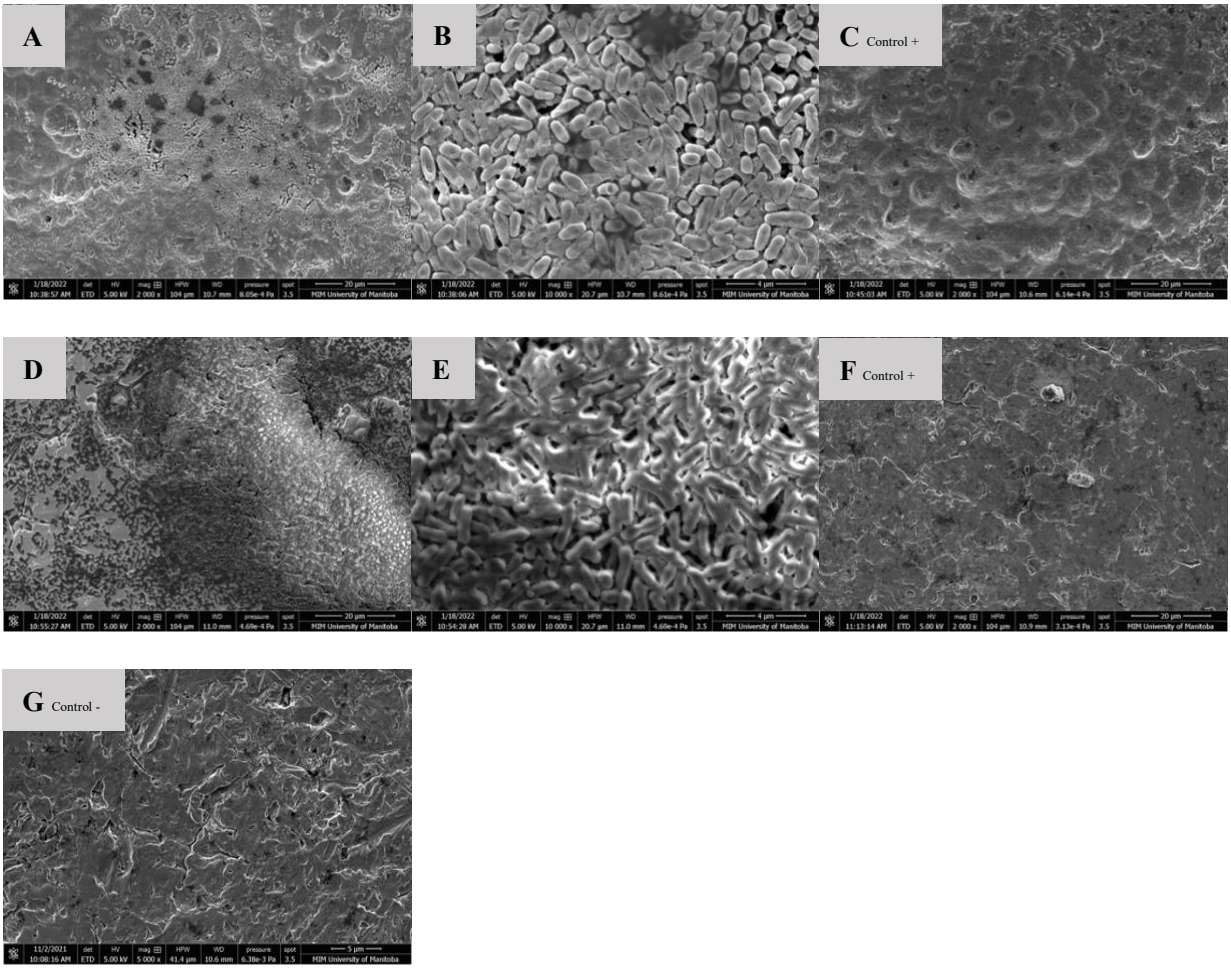

Figure 3-5. SEM of A) to B) T2: *C. koreensis* + *R. terrigena*; and C) T4: STEC O103:H2 Control positive 10 °C dry biofilms at day 60 on a TPU surface. D) to E) T2: *C. koreensis* + *R. terrigena*; and F) T4: STEC O103:H2 Control positive 10 °C dry biofilm at day 60 on a SS surface; G) SS coupon used as control negative, no bacteria observed.
In panels **A**), **B**), **D**), and **E**) 60 d dry biofilms are shown on TPU and SS surface, the biofilm is dominated by rod-shaped bacteria with the EPS matrix; a well-developed multilayered biofilm displayed, which covered the TPU and SS surface. In panels **C**) and **F**) no individual bacteria and EPS were displayed on the surface of TPU and SS coupons.

3.5. Discussion

3.5.1. Biofilm Forming Ability

In this study, strains isolated from beef processing facilities (e.g., *L. sakei* S19, *C. maltaromaticum,* and *R. terrigena*) and meat products (*Serratia* sp.) were found to be stronger

biofilm formers at 10 °C than those (e.g., *L. bulgaricus* and *L. curvatus*) isolated from the fermented product. This is showing that isolates from beef production facilities are likely adapted to growth and form biofilms at low temperatures (Visvalingam et al., 2019a). In Canada, beef fabrication facilities operate at temperatures below 10 °C to limit the growth of enteric pathogens and spoilage bacteria (Visvalingam et al., 2017a; Yang et al., 2017a; Yang et al., 2017b). However, temperatures at the beef fabrication facility vary and reach up to 15 °C during nonoperational hours (Visvalingam et al., 2017a). In addition, the equipment used during beef fabrication and other interventions can generate higher temperatures, for example, the frictional heat produced by conveyor belts, the transfer of body heat to gloves, and application of high-pressure hot water (40 -50 °C) during sanitation (Yang et al., 2017a; Wang et al., 2018). Thus, temperature variation and microenvironments created in the beef processing facility attributed to different factors could promote the formation of biofilms within the beef processing environment (Yang et al., 2015; Visvalingam et al., 2016; Yang et al., 2017a).

3.5.2. STEC Curli and Cellulose Production Determination

Curli fimbriae and the production of cellulose have been reported to play a significant role in STEC biofilm formation and persistence (Gualdi et al., 2008; Iibuchi et al., 2010; Adator et al., 2018). Curli plays a significant role in mediating surface and cell to cell contact in *E. coli* & *Salmonella* biofilms (Barnhart and Chapman, 2006). Uhlich et al. (2014) demonstrate that curli and cellulose formation are influenced by temperature and media composition (Uhlich et al., 2014). Results in this research demonstrate STEC strain O145:H2 (75-83) and O157:H7 (1934) showed curli producing ability at 37 °C instead 25 °C, while only STEC O157:H7 (R508) and *E. coli* (8_77) kept cellulose producing capacity at 37 °C (Table 8-1). With the exception of the strong biofilm former O157:H7 (1931), STEC that lacked curli or did not produce cellulose were only able to form weak or intermediate biofilms at 25 °C (Table 3-3). Similar results were reported by Wang et al. (2012), where some STEC strains that lacked curli could still produce strong biofilms at room temperature (Wang et al., 2012a). Stanford et al. (2021) also found that some STEC strains that

produced curli did not form strong biofilms. This indicates that traits other than just curli and cellulose production are likely to mediate biofilm formation (Wang et al., 2012a; Visvalingam et al., 2017a).

3.5.3. Contamination of Food Contact Surfaces by Multispecies Biofilms

Some studies have shown that *E. coli* found on the surface of fabrication equipment can survive sanitation procedures and contaminate meat (Yang et al., 2015; Visvalingam et al., 2016). STEC attached to food contact surfaces might also interact with pre-established multispecies biofilms (Wang, 2019). In the present study, well-structured multilayer multispecies biofilms were developed (Figure 3-4 and 3-5) and O103:H2 within these biofilms was able to transfer to beef (Figure 3-3), Furthermore, Visvalingam et al. (2019) demonstrated that STEC O157:H7 readily integrated (3.8 $\log_{10}$ CFU/cm^2) into multispecies biofilms containing 41 different bacterial strain isolated from beef packing plant (Visvalingam et al., 2019a). In the same study, the relative abundance of participating strains (n = 42) did vary; as some strains such as *Carnobacterium* sp. accounted for (10%) of the biofilm membership whereas O157:H7 accounted for only 0.04 % of the community (Visvalingam et al., 2019a). Thus, for the multispecies biofilm formed on food contact surfaces in this study, we were not looking into biofilm composition, the target was to get information on bacterial associations and cross-contamination potential.

Contamination of beef by O103:H2 was substantially reduced after interaction with *P. aeruginosa* + *C. koreensis* (T3) biofilms. Both *Pseudomonas* sp. (Pang et al., 2017; Pang and Yuk, 2018) and *Comamonas* sp. (Carpentier and Chassaing, 2004) have been shown to form robust biofilms on stainless steel surfaces. In the present study, pre-existing biofilms of *P. aeruginosa* + *C. koreensi*s on TPU and SS may have inhibited O103:H2 integration into biofilms. Wang et al. (2015) investigated mixed biofilms of STEC serotypes O157:H7 and O111:H8 and found that the STEC serotype that was inoculated onto the surface first, exhibited the dominant membership within mature biofilms (Wang et al., 2015). Most biofilm studies have inoculated similar numbers of different bacterial species onto food contact surfaces (Wang et al., 2013b; Liu et al., 2014; Pang

et al., 2017). Within beef fabrication facilities, it is likely that multispecies biofilm is already established on contact surfaces (Wang, 2019). These mature biofilms may preclude the integration of STEC due to a lack of adhesion sites or available nutrients (Pang and Yuk, 2018). Hence, developing biofilms composed of pre-selected innocuous bacterial species may inhibit STEC biofilm formation and have merit as a biocontrol strategy (Alegre et al., 2013). In the other hand, *C. piscicola* + *L. bulgaricus* (T1) and *C. koreensis* + *R. terrigena*. (T2) biofilms did not affect the extent that O103:H2 contaminated beef. The impact of mixed-species biofilms on the contamination of meat is likely species and possibly strain-dependent (Wang, 2019). For example, *Pseudomonas* sp. have been shown to inhibit the formation of *E. coli* O157:H7 biofilms on SS (Kim et al., 2018) and *Salmonella* biofilms on TPU (Wang et al., 2013a). *Pseudomonas* sp. is known to produce antimicrobials such as pyocyanin, pyoluteorin, and siderophores, which may inhibit the integration of foreign bacteria into biofilms (Hernández-León et al., 2015; Collazo et al., 2017; Kim et al., 2018). Moreover, *P. aeruginosa* may also produce acyl-homoserine lactones (AHL) (Lee et al., 2007), which have been shown to inhibit *E. coli* biofilm formation by altering gene expression (Van Houdt et al., 2006).

3.5.4. Interactions of LAB Multispecies Biofilms with O103:H2

Certain groups of LAB such as *Lactobacillus* sp. are commonly used as probiotics, as many of these isolates produce bacteriocins and organic acids (e.g., lactic acid) (Schrezenmeir and De Vrese, 2001; Imani Fooladi et al., 2014). Previous research indicates that some *Lactobacillus* spp. can reduce the shedding of *E. coli* O157:H7 in cattle feces (Brashears et al., 2003; Younts-Dahl et al., 2004). If added to ground beef at 5 °C, they can also reduce the prevalence of *E. coli* O157:H7 and *Salmonella* (Smith et al., 2005). *Carnobacterium* sp. are frequently isolated from beef fabrication facilities, and can persist on non-food-contact surfaces after sanitization (Wang et al., 2018). Interestingly, multiple studies indicate *Carnobacterium* sp. can inhibit the growth of *Listeria monocytogenes* on meat by producing bacteriocins, but this species can also cause food spoilage (Leisner et al., 2007). In the present study, no synergistic or antagonistic interactions on

beef contamination were observed between O103:H2 and *C. piscicola* + *L. bulgaricus* biofilms. Furthermore, extracts of *C. piscicola* and *L. bulgaricus* did not exhibit activity against O103:H2 in clearing zone assays (data not shown). Most LAB bacteriocins target a narrow range of bacteria and primarily target gram-positive bacteria such as *Listeria* (Jones et al., 2008). Jones et al. (2008) tested 75 meat-related LAB strains and none of them showed antimicrobial activity against *E. coli* O157:H7 (Jones et al., 2008). Others have shown that *Lactobacillus* sp. and *Lactococcus* sp. bacteriocin activity against *E. coli* is strain-dependent (Gómez et al., 2016). *Lactobacillus* sp. can also produce lactic, acetic, and propionic acids, which can inhibit the growth of pathogenic bacteria (Abedi et al., 2013; Jalilsood et al., 2015). However, after 6 d the alkaline extract (pH > 8) was produced by *C. piscicola* + *L. bulgaricus* (T1) biofilms, suggesting that the production of ammonia from amino acid metabolism may have neutralized any antimicrobial activity of organic acids.

3.5.5. Beef Contamination by O103:H2 Varies on Different Food Contact Surface

Stainless steel and thermoplastics are two of the most common food contact surfaces used in the food industry (Chia et al., 2009; Sofos and Geornaras, 2010). In this research, the transfer of O103:H2 to beef from biofilms on TPU was greater than from SS (Figure 3-3). In previous studies, *E. coli* O157:H7 and *L. monocytogenes* were able to form stronger monoculture biofilms (higher bacteria count) on polyurethane plastic than stainless steel (SS-304) (Midelet and Carpentier, 2002; Graziella et al., 2006; Sofos and Geornaras, 2010). The stronger biofilms on polyurethane surfaces may be related to its greater hydrophobicity than stainless steel (Donlan, 2002). It has been hypothesized that as bacteria cells irreversibly attach to solid surfaces, hydrophobic surfaces may have less electrostatic repulsive forces (Loosecht et al., 1987; Sinde and Carballo, 2000; Donlan, 2002). Other bacteria such as *Salmonella* and *Listeria* have also been found to more readily attach and form biofilms on surfaces that are more hydrophobic (Sinde and Carballo, 2000; Donlan, 2002). Apart from beef contamination, the different food contact surfaces also affected O103:H2 recovery from 60 d dry biofilms (Table 3-5), with O103:H2 being more readily recovered from multispecies on TPU than SS at both 10 and 25 °C. A similar result has been reported by Adator et

al. (2018), as recovery rates from STECs biofilms were higher on polystyrene than SS (Adator et al., 2018). Previous studies have demonstrated that conveyor belts are often linked to the contamination of beef trimmings and cuts with *E. coli* in beef fabrication facilities (Youssef et al., 2013; Yang et al., 2015; Visvalingam et al., 2016). Regarding the efficiency of routine commercial sanitation processes in beef processing facilities, published work shows that the sanitation process cannot completely remove *E. coli* from the conveyor belt (Yang et al., 2015; Visvalingam et al., 2016; Yang et al., 2017b). This limited *E. coli* removal has been attributed to meat residues, which can reduce the efficacy of sanitizers and the impact of desiccation on the viability of *E.coli* (Yang et al., 2015; Visvalingam et al., 2016; Yang et al., 2017b). We found that O103:H2, within 60 d, dry biofilms did not transfer to beef (Figure 3-3). In contrast within the moist biofilm, O103:H2 bacteria readily transferred to beef even after 60 d of storage at 10 or 25 °C (Figure 3-3). As indicated by Gill and Lander (2004), others have shown that desiccation can reduce the transfer of *E. coli* from fabrication equipment to beef (Gill and Landers, 2004; Youssef et al., 2013). However, humid conditions are prevalent in the beef industry due to condensation originating from the routine use of hot water during the sanitation processes (Møretrø et al., 2010), which could re-hydrate dry biofilms allowing bacterial within the biofilm to thrive, persist and spread. Typically, the relative humidity within beef processing plants is high, varying from 40 to 97% during the day with peak humidity's reached during sanitation (Møretrø et al., 2010). Meat residues on the conveyer belts, combined with the high relative humidity are factors that undoubtedly contribute to the formation of robust surface biofilms (Yang et al., 2015; Visvalingam et al., 2016; Yang et al., 2017b) or maintenance of dry old biofilms. Results obtained in this research showed that prolonged storage time was associated with a decrease in the transfer of O103:H2 from biofilms to beef at both 10 and 25 °C. During prolonged storage, nutrients within biofilms may become limiting and a buildup of waste products may also reduce cell viability within biofilms (Kumar and Anand, 1998a; Rendueles and Ghigo, 2015). However, dry biofilms can pose a cross-contamination risk if those dry biofilms become in contact with meat juices and water since STEC can still be viable within the biofilm.

3.5.6. The O103:H2 persistence in dry multispecies biofilm during long periods of storage

Although O103:H2 associated with 60 d dry biofilms did not transfer to beef, viable O103:H2 could still be recovered from dry biofilm after enrichment (Table 3-5). Others have also found that dormant STEC cells can be recovered from dry biofilms via enrichment which mimics the conditions at the meat plants where dry-biofilms could rehydrate with water and beef juices allowing bacteria to recover (Adator et al., 2018). After enrichment, O103:H2 recovery from *C. koreensis* + *R. terrigena* (T2) mixed biofilm at 10 °C was always higher than from positive control (T4) 60 d dry biofilms (Table 3-4). Furthermore, SEM images indicated that 10 °C *C. koreensis* + *R. terrigena* (T2) 60 d dry biofilm displayed well-structured multilayered biofilm on TPU and SS surfaces (Figure 3-5). Instead, 10 °C positive control (T4) 60 d dry biofilm was non-observed on both surfaces (Figure 3-5). This is showing that spoilage bacteria naturally occurring in beef processing environments, who could survive the sanitation process and are adapted to lower temperatures, could shelter STEC and allow its persistence. Other researchers have made similar observations for *Pseudomonas – Salmonella* mixed biofilms, where the presence of *P. aeruginosa* enhanced *Salmonella typhimurium* and *Salmonella enteritidis* resistance to sanitizers (Pang et al., 2017). *P. aeruginosa* has been shown to produce more EPS (e.g., glycoconjugates) in mixed-species biofilms, thicker EPS hinder sanitizer penetration and thus protect *Salmonella* against sanitizers (Pang et al., 2017; Pang and Yuk, 2018). During biofilm development, EPS are secreted by the bacterial community, complex matrixes are formed which in turn embeds bacterial cells (Flemming and Wingender, 2010) and protects them from desiccation while trapping nutrients (Kumar and Anand, 1998a; Stewart and Franklin, 2008). Therefore, it's possible that *C. koreensis* + *R. terrigena* (T2) produced a more complex EPS matrix at 10 ºC (Figure 3-5) that enhanced the ability of O103:H2 to persist in desiccated multispecies biofilms. In beef processing facilities, dry biofilms on beef fabrication equipment that come in contact with beef purge or water may result in conditions that promote cell viability like enrichment (Skandamis et al., 2009; Adator et al., 2018). If this is the case, even old dry biofilms on beef fabrication equipment could continuously

pose a risk to beef contamination (Skandamis et al., 2009; Yang et al., 2017a; Adator et al., 2018).

Moreover, a low environmental temperature (5-15 °C) is maintained in the beef industry to limit enteric pathogen growth on food contact surfaces and the product (Ma et al., 2019; Ma et al., 2020). And 10 ºC reduced the O103:H2 biofilm formation and cell transfer to beef than 25 ºC in the present studies. However, a higher O103:H2 recovery rate was observed in 60 d dry biofilms formed and maintained at 10 ºC instead of 25 ºC, especially the highest O103:H2 recovery was identified from *C. koreensis* + *R. terrigena* (T2) mixed biofilm at 10 °C. This finding suggests that despite reducing STEC biofilm formation in beef fabrication facilities through temperature control, STEC can still pose a cross-contamination risk to beef, especially under the assistance of spoilage bacteria in the beef processing environment.

3.6. Conclusions

Bacteria commonly found in the food industry played a significant role in STEC persistence and survival. The biofilm mixture *P. aeruginosa* + *C. koreensis* was the most antagonistic towards O103:H2 at 25 °C. And *C. koreensis* + *R. terrigena* dry biofilms showed the highest recovery of O103:H2 at 10 °C. Moreover, LAB biofilm did not reduce the extent to which O103:H2 was transferred from biofilms to beef, which may indicate that the interaction between O103:H2 and pre-developed biofilm was species-dependent. Conditions for multispecies biofilm formation, including humidity, adherent surface, and storage time are variables, that played significant roles in beef contamination by O103:H2. Beef contamination with O103:H2 was more severe when it contacted fresh moist biofilms on TPU. Thus, further improvements for cleaning conveyor belts should be explored since scientific data is indicating that conveyor belts material allows biofilm formation and persistence. Perhaps developing different materials less prone to bacterial attachment and colonization should be explored. Further, O103:H2 biofilm formation reduced at low temperatures; however, a higher STEC recovery from 10 °C dry biofilms was observed. Results suggest STEC persistence may not only depend on biofilm-forming ability but also be related to the bacteria community in the beef processing environment. Findings in the present study

confirm that development of SP or LAB multispecies biofilms with O103:H2 can either increase or diminish the likelihood of beef contamination. Further studies could attempt to investigate the general interaction between SP or LAB multispecies biofilm with STEC (e.g., top 7 STEC) and also should look into the genetic makeup of STEC. Such work could facilitate the development of biofilm management strategies for STEC in beef processing environments.

4. Chapter 4: Influence of lactic acid and spoilage bacteria on *E. coli* O157:H7 biofilms on food contact surfaces and their transfer to beef.

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4.1. Abstract

Biofilm formation is one of the mechanisms that Shiga toxin-producing *Escherichia coli* (STEC) could use to persist and spread in the beef fabrication facility. We investigated the interaction between STEC and selected lactic acid bacteria (LAB) or spoilage bacteria (SP) within multispecies biofilms on polyurethane (TPU) and stainless steel (SS) surfaces after storage by 6, 30, and 60 d under wet and dry conditions at 10 and 25 ℃. The ability of O157:H7 to form multispecies biofilms with one LAB (**T1)**: *Carnobacterium piscicola* + *Lactobacillus bulgaricus,* and two SP (**T2)**: *Comamonas koreensis* + *Raoultella terrigena*; (**T3)**: *Pseudomonas aeruginosa* + *C. koreensis* strain combination was investigated. O157:H7 single-species biofilms were treated as a control (**T4**). The O157:H7 transfer to beef and survival ability was assessed after storage for up to 60 d under dry (20%-50% relative humidity; RH) or moist (60~90% RH) conditions. Regardless of temperature, a greater extent of O157:H7 viable cell transfer to beef from fresher (6 d), moist biofilm, on the TPU surface was observed. On the other hand, we did not detect O157:H7 on beef after contact with 30 and 60 d old dry single species biofilm (T4) of the organism. However, O157:H7 was still recovered from multispecies biofilm through enrichment. At 25°C, T3 reduced O157:H7 cell transfer to beef by 1.76 $\log_{10}$ CFU/g ($P < 0.001$). On TPU surfaces, the recovery rates for O157:H7 from T1-dry and T2-dry 60 d biofilm were 67% and 22%, respectively; while on SS surfaces the recovery was 0% (T1-dry) and 11% (T2-dry). *E. coli* O157:H7 was not recovered on any of the tested surfaces from T3-dry and T4-dry biofilm at 25 ℃. At 10°C, the overall beef contamination with O157:H7 was similar among all strain combinations (vary from 0.94 to 1.38 $\log_{10}$ CFU/g) ($P > 0.05$). On the TPU surface, after enrichment of the 60-d old dry biofilm, O157:H7 was recovered from 89% of T2-dry and T3-dry biofilms. On SS the recovery was 22% (T2-dry) and 0% (T3-dry). Interestingly, no O157:H7 was recovered (0%) from T1-dry and T4-dry biofilm on any of the tested surfaces. Results demonstrate that multiple environmental factors such as contact surface, biofilm age, humidity, and presence of other bacteria, can affect the risk of beef contamination. Multispecies biofilm developed in this study enhanced O157:H7 transfer to beef and persistence in dry and wet biofilm at different temperatures.

4.2. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are a group of *E. coli* that produce Shiga toxins and are important enteric pathogens worldwide (Nguyen and Sperandio, 2012; CDC, 2014a). Shiga toxins secreted by STEC include both Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), encoded by stx_1 and stx_2 (Padola and Etcheverria, 2014). By producing Stx1 and Stx2, serious complications such as Hemolytic Uremic Syndrome and Hemorrhagic Colitis can be caused by a low infectious dosage (ten cells) (Etcheverria et al., 2010; PHAC, 2015). In Canada, non-O157 serogroups related to foodborne diseases are higher than O157-related since 2017 (PHAC, 2020). In 2019, 1,462 cases of STEC infections were reported to the Canadian National Enteric Surveillance Program (NESP); only 27% were caused by O157 STEC (PHAC, 2020). However, O157:H7 was still the most common STEC subtypes isolated from clinical cases (34.48%) in 2018, followed by O26:H11 (11.49%), O111:NM (5.75%), and O103:H2 (5.75%) (PHAC, 2019). Therefore, the O157 is one of the major food safety concerns in the food industry due to its high pathogenicity (Noftall et al., 2019).

Ruminants are considered the main STEC reservoir (Bryan et al., 2015). STEC can be present in the animal feces and contaminate the hides, during slaughter STEC from hides or feces can contaminate the beef carcasses as well as beef primal and sub-primal (Bryan et al., 2015; PHAC, 2015). Microbial testing in Canadian beef processing facilities has shown a sporadically high positive rate for STEC contamination, which is referred to as high event periods (HEP), and the causes of HEP are unknown (Stanford et al., 2017). A recent study suggested that biofilm formation might play a significant role in the HEP phenomenon (Wang et al., 2014; Wang et al., 2016b). Biofilm is defined as a community of microorganisms that adhere to the surface and harboured within a self-produced Extracellular Polymeric Substance (EPS) (Srey et al., 2013; Vogeleer et al., 2014; Adator et al., 2018). Arthur et al. (2014) collected a total of 639 beef trim samples from 21 cases of O157:H7 related HEP and noticed that each HEP event was dominated by a single O157:H7 strain type (Arthur et al., 2014). Moreover, the O157:H7 strains isolated from HEP beef

products have demonstrated strong biofilm-forming ability and resistance towards sanitizers under biofilm form (Wang et al., 2014). HEP events can result in significant economic losses due to beef product recall and product destruction (FSIS, 2014; Wang et al., 2016b). Some research has reported the biofilm-forming ability of STEC isolates (n=745) from cattle (Stanford et al., 2021), it was reported that only one STEC strain out of 745 was able to form strong biofilms. Strong biofilm formers are more likely to cause long-term environmental contamination that might result in beef contamination (HEP). Biofilms can prevent sanitizers from coming in contact with the bacteria inside, resulting in bacteria surviving the sanitation process and its persistence on the beef fabrication equipment (Stanford et al., 2021).

A study by Adator et al. demonstrated STEC in dry single species biofilms could survive after one month's storage and cause lettuce contamination (Adator et al., 2018). Understanding the STEC interaction with biofilms containing multiple microorganisms could fill knowledge gaps that could lead to enhancing pathogen biocontrol strategies in the food industry (Burmolle et al., 2014; Rendueles and Ghigo, 2015). In the beef processing facility, lactic acid bacteria (e.g., *Carnobacterium* sp.) and spoilage bacteria (e.g., *Raoultella* sp.) were able to persist on beef fabrication equipment such as conveyor belts (Wang et al., 2018). Besides, previous studies have shown that lactic acid bacteria can develop biofilms on food contact surfaces or drainage systems, which has been used as an efficient method for *Listeria* elimination in the food industry (Zhao et al., 2004; Castellano et al., 2008; Pérez Ibarreche et al., 2014). Thus, those LAB bacteria and spoilage bacteria that can persist on the food contact surfaces could eventually form multispecies biofilm and interact with STEC (Giaouris et al., 2015). To develop risk assessment tools and to develop pathogen reduction interventions, it is important to investigate the interactions between pathogenic bacteria such as O157:H7 and other bacterial strains (e.g., LAB) that could be present in the food processing environment and could potentially form biofilm and to study their interactions (Giaouris et al., 2015). The general aim of this research was to explore *E. coli* O157:H7 interactions with other bacterial species commonly found in beef processing environments as well as the role of several environmental variables on STEC biofilm formation. The specific objectives

were as follows (1) evaluate potential synergistic and antagonism interactions of O157:H7 within either selected LAB or SP multispecies biofilm on thermoplastic polyurethane (TPU) and stainless steel (SS) surfaces at different storage times, temperatures, and humidity; (2) to determine the extent of O157:H7 transfer from single and multispecies biofilms to beef under these conditions; and (3) to determine the survival capacity of O157:H7 within single and multispecies biofilms.

4.3. Material and Methods

4.3.1. Bacteria Strains and Culture Conditions

One STEC O157:H7 strain, 3 SP, and 2 LAB strains were tested in this study (Table 4-1). All bacterial strains were stored at -80 ℃ in Lysogeny Broth with no salt (LB-NS; Tryptone 10 g/L and Yeast extract 5 g/L) containing 15% glycerol. The SP and LAB strains were incubated on Trypticase Soy Agar (TSA; Becton, Dickinson and Company, Sparks, USA), while STEC strains were incubated on MacConkey agar plates (Hardy Diagnostics Inc., Santa Maria, USA) at 25 ℃. One individual colony from each culture was picked from each agar plate and transferred into 10 mL LB-NS, which was incubated at the designated time and temperature for each culture to reach a 10^8 CFU/mL cell concentration. The time required for each strain to achieve an early stationary phase varied from 24 to 72 hours (Visvalingam et al., 2019a). Each culture was subsequently used in biofilm development.

To mimic beef fabrication environments, the sterile beef purge was supplemented into LB-NS broth (Pang and Yuk, 2018). To maintain the consistency of beef purge between different batches, the same brand of vacuum-packed beef (ie. eye of round beef cuts with a fat cap) was purchased for each experiment. Briefly, the beef purge (blood) was collected once the vacuum package was opened, then it was diluted with distilled water at a ratio of 1:6. The diluted beef purge was then sterilized by filtering through a 0.45 μm sterile filter (Midelet and Carpentier, 2002). The protein content was evaluated using a Coomassie (Bradford) protein assay kit (Thermo Scientific, Rockford, IL), (Kruger, 2009). The diluted sterile beef purge (14.3 %) was kept at -

20 °C and added into LB-NS broth (10% v/v; mLB-NS) before use.

Table 4-1. STEC, LAB, and SP bacteria selected for multispecies biofilm development.

Serotype	Strain ID	Source	Category	Biofilm at 25 ℃	Biofilm at 10 ℃
O157: H7	1934	Beef	STEC	Intermediate	Weak
Lactobacillus bulgaricus	ATCC11842	Yogurt	LAB	Strong	Weak
Carnobacterium piscicola	M5L1	Vacuum package pork	LAB	Intermediate	Weak
Comamonas koreensis	25_64	Meatpacking plant	Spoilage	Weak	Weak
Raoultella terrigena	ENT25_16	Meatpacking plant	Spoilage	Strong	Strong
Pseudomonas aeruginosa	ATCC 7700	Well water	Spoilage	Strong	Weak

4.3.2. Multispecies Biofilm Formation on Food Contact Surface and O157:H7 Transfer to Beef

4.3.2.1. Bacterial Strain and Culture Combination

The following three strain-combinations were used to investigate the ability of O157:H7 (1934) to form multispecies biofilm on the food contact surfaces and to contaminate the beef product, which included: one LAB combination, **T1**: *Carnobacterium piscicola* + *Lactobacillus bulgaricus,* and two SP combinations **T2**: *Comamonas koreensis* + *Raoultella terrigena* and **T3**: *Pseudomonas aeruginosa* + *C. koreensis*. *E. coli* O157:H7 (strain 1934) single-species biofilms were included as a positive control (**T4**). To prepare the LAB/ SP bacterial biofilms, methods from Wang et al. 2013 were followed with some adjustments (Wang et al., 2013b). Generally, fresh cultures of each LAB and SP strain were diluted with mLB-NS to achieve a bacterial concentration of 10^6 CFU/mL, then the diluted cultures were mixed respectively according to the experiment design.

4.3.2.2. Polyurethane and Stainless-steel Coupons Preparation

Thermoplastic polyurethane coupons were prepared as described by Dourou et al. 2011 with some modifications (Dourou et al., 2011). Briefly, the TPU conveyor belt (2E8U 0/02 White, NuTech Conveyor Components, Milton, USA) was cut manually into 2 cm × 2 cm pieces and soaked in hydrogen peroxide (Accel® PREVention$^{TM/MC}$, Diversey^{TM}, Fort Mill, USA) overnight for sanitization. The TPU coupons were then washed with sterile distilled water for 1 hour (Dourou et al., 2011).

Stainless steel 304 coupons (2 cm- diameter; Pegen Industries Inc., Stittsville, CA) were washed with distilled water and sonicated in an ultrasonic water bath for 20 minutes at 60℃ (Adator et al., 2018). Then, the SS-304 coupons were sonicated in phosphoric acid solution (15% v/v) for 20 minutes at 60℃, then sonicated in distilled water for 20 minutes and dry-sterilized in an autoclave before the experiment.

4.3.2.3. Dry and Wet Multispecies Biofilm Formation

Coupons were placed into sterile Petri dishes (60 x 15 mm; VWR^{TM}, Radnor, USA) and allocated to different treatments. Then 5 mL of either LAB or SP bacterial cultures (10^6 CFU/ml) combination was transferred into each designated coupon (Adator et al., 2018). Coupons were stored under temperatures of either 10 or 25℃, for 6 d to develop mature biofilms. On d 6, the coupons were then washed with BPB three times (10 mL/ coupon) and transferred in a new sterile Petri Dish. Then, aliquots (5 mL) of the STEC O157:H7 culture (10^3 CFU/mL) were added to the preformed biofilms and incubated for another 6 days in the same condition (10 and 25℃). The O157 single-species biofilm was developed as a control positive (**T4**). Coupons that received no inoculant were treated as a negative control. The coupons were washed as previously described and dried for 4 hours at room temperature in a BSL2 cabinet. All the coupons were stored at moist conditions (60~90% relative humidity, (RH)) or dry (20~50% RH) at 10 & 25 °C. Moist biofilms were sprayed with sterile water (150 µL/ coupons) once per day. A subgroup of SS and TPU

coupons were selected to test the capacity of STEC transfer to beef. The second group of coupons was chosen for STEC enumeration form biofilm on d 6, 30, and 60.

4.3.2.4. Beef Samples Preparation to Test O157 Transfer

The vacuum-packed eye of round beef whole cuts (with a fat cap) was purchased and stored at 4 °C and used within one week. The meat surface was washed and soaked within 5% lactic acid solution for 1 minute to decrease the background flora on the beef surface (Youssef et al., 2013). After the immersion in lactic acid for 1 minute, the meat pieces were drained for 1 minute. The beef was properly drained and cut into 3 cm x 3 cm lean beef pieces under sterile conditions and kept at 4 °C for less than 24 hours. *E. coli* presence on beef was evaluated by randomly selecting three beef pieces and individually placed into a Whirl-Pak bag (Nasco®; Madison WI, USA). Each bag containing 9 mL of Buffered peptone water (BPW, Hardy Diagnostics Inc.) for ten-fold dilution and homogenized using a stomacher for 1 minute (Intersciences Inc., Markham, Canada) and plating on MacConkey agar.

4.3.2.5. STEC Transfer from Biofilms to Beef and STEC Biofilm Enumeration

For O157:H7 transfer, beef pieces (3 x 3 cm) were placed on top of the TPU and SS coupons. A 50 g weight was placed on top of the beef pieces to provide pressure (7.35 kPa) (Flores et al., 2006). To avoid direct contact with the weight, the wax paper piece was placed between the weight and the meat. After 5 minutes' contact, the beef pieces were collected from each coupon and placed into a Whirl-Pak bag each containing 9 mL of Buffered peptone water for 1 minute homogenization using a stomacher. For O157:H7 enumeration, 10-fold dilutions were performed and plated on TSA overlayed MacConkey agar (Wu, 2008). The TSA overlay's purpose was to enhance potentially stressed O157:H7 cells to recover (Medina et al., 2020). Plates were incubated for 24 hours at 37 °C. Presumptive *E. coli* colonies were confirmed as STEC O157 by serological (O-antigens) agglutination test (SSI Diagnostica, Hiller.d, Denmark) and PCR (Adator et al., 2018).

To quantify O157:H7 on TPU and SS coupons, the coupons were transferred into Whirl-Pak bags along with 9 mL of BPW to achieve ten-fold dilution and then sonicated for 1 minute (Marouani-Gadri et al., 2009). For O157:H7 enumeration the drop plate method was used as to described above. Samples not showing STEC colonies on MacConkey plates (undetectable levels) after 24 hours incubation at 37°C, were enriched with modified tryptone soy broth (mTSB; Oxoid Ltd., Nepean, Canada) for 24 hours at 37°C then spread on MacConkey agar.

4.3.3. Scanning Electron Microscopy

Based on the ability of O157:H7 to transfer to beef and to survive within the dry biofilm, multispecies biofilm *C. koreensis* + *R. terrigena* (T2) and *P. aeruginosa* + *C. koreensis* (T3) were further investigated by Scanning electron microscopy (SEM). Scanning electron microscopy (SEM) was performed at the Manitoba Institute for Materials (MIM) to observe dry biofilm formation on TPU and SS surfaces as previously described (Adator et al., 2018). The TPU and SS coupons were fixed in 10% formalin solution (neutral buffered; Sigma Aldrich) for 2 hours and then washed and immersed in BPB for 30 minutes. The coupons were air-dried for 4 hours at room temperature, and the TPU surfaces were coated with Gold-Palladium (Denton Vacuum Desk II, Moorestown, US). Biofilm structures were visualized using a QuantaTM 650 FEG scanning electron microscope (FEI CO., Hillsboro, US) at 5 KV in the high-vacuum mode.

4.3.4. Statistics Analysis

All experiments were performed three times independently. The Statistical Analysis System Proc Mixed program (Cary, USA) was applied to perform the statistical analysis by using the least mean separation accomplished using the PDIFF option. For beef contaminated by O157:H7 biofilm from food contact surface, effects of the contact surface, storage time, species, and humidity along with associated interactions were tested. For all statistical analyses, a significance level of $P \leq 0.05$ was applied.

4.4. Results

4.4.1. Multispecies Biofilm Formation on Food Contact Surface and O157:H7 Transfer to Beef

4.4.1.1. O157:H7 Transfer to Beef at 25 °C

At 25 °C, the spoilage biofilm combination *P. aeruginosa* + *C. koreensis* (T3) reduced ($P < 0.001$) O157:H7 cell transfer to beef by 1.76 $\log_{10}$ CFU/g (Figure 4-1. A, B, C). While the overall beef contamination by *C. piscicola* + *L. bulgaricus* (T1) and *C. koreensis* + *R. terrigena* (T2) biofilm mixtures showed similar cell transfer ($P > 0.05$) when compared with the control positive (T4).

As to surface type, overall O157:H7 beef contamination from TPU (2.22 $\log_{10}$ CFU/g) was higher ($P < 0.001$) than from SS (1.54 $\log_{10}$ CFU/g). Regarding biofilm aging time, the overall beef contamination with O157:H7 decreased with biofilm aging time, from 3.07 log (6 d) to 1.74 logs (30 d) and 0.83 logs (60 d) ($P < 0.001$). The 6-d biofilm mixtures *P. aeruginosa* + C. *koreensis* (T3) on the TPU surface showed the highest antagonistic effect on O157 transfer to beef (2.80 $\log_{10}$ CFU/g). Meanwhile, the highest beef contamination with O157:H7 was caused by 6 d *C. koreensis* + *R. terrigena* (T2) biofilm on TPU surfaces (4.99 $\log_{10}$ CFU/g). After 60 d storage, no O157:H7 transfer to beef from *P. aeruginosa* + C. *koreensis* (T3) biofilm mixture was detected, and beef contamination with O157:H7 from *C. koreensis* + *R. terrigena* (T2) on SS surface (0.14 $\log_{10}$ CFU/g) was lower ($P < 0.05$) than control positive (T4)'s (1.19 $\log_{10}$ CFU/g). Overall moist biofilms caused higher ($P < 0.001$) beef contamination (3.50 $\log_{10}$ CFU/g) when compared with dry biofilms (0.26 $\log_{10}$ CFU/g).

4.4.1.2. O157:H7 Transfer to Beef at 10 °C

At 10 °C, none of the tested multispecies biofilms combinations affected the beef contamination with O157:H7 when compared with control positive (T4), which ranged from 0.94 – 1.38 $\log_{10}$ CFU/g ($P > 0.05$) (Figure 4-1. D, E, F). As per the surface type, similarly to what was observed at 25 °C, the transfer of O157:H7 to beef was greater ($P < 0.001$) from the TPU surface (1.42 $\log_{10}$ CFU/g) than the SS surface (0.82 $\log_{10}$ CFU/g). Regarding humidity conditions, the overall beef contamination with O157:H7 from moist biofilms (2.20 $\log_{10}$ CFU/g) was higher ($P < 0.001$) than contamination from the dry biofilms (0.05 $\log_{10}$ CFU/g). As the biofilms aged, the O157:H7 cell transfer to beef decreased ($P < 0.001$). The transfer was as follows, at day 6, 1.79 $\log_{10}$ CFU/g, day 30, 0.95 $\log_{10}$ CFU/g and at day 60, 0.63 $\log_{10}$ CFU/g. No transfer of O157:H7 cell from dry biofilm after 30 and 60 d storage to beef was detected.

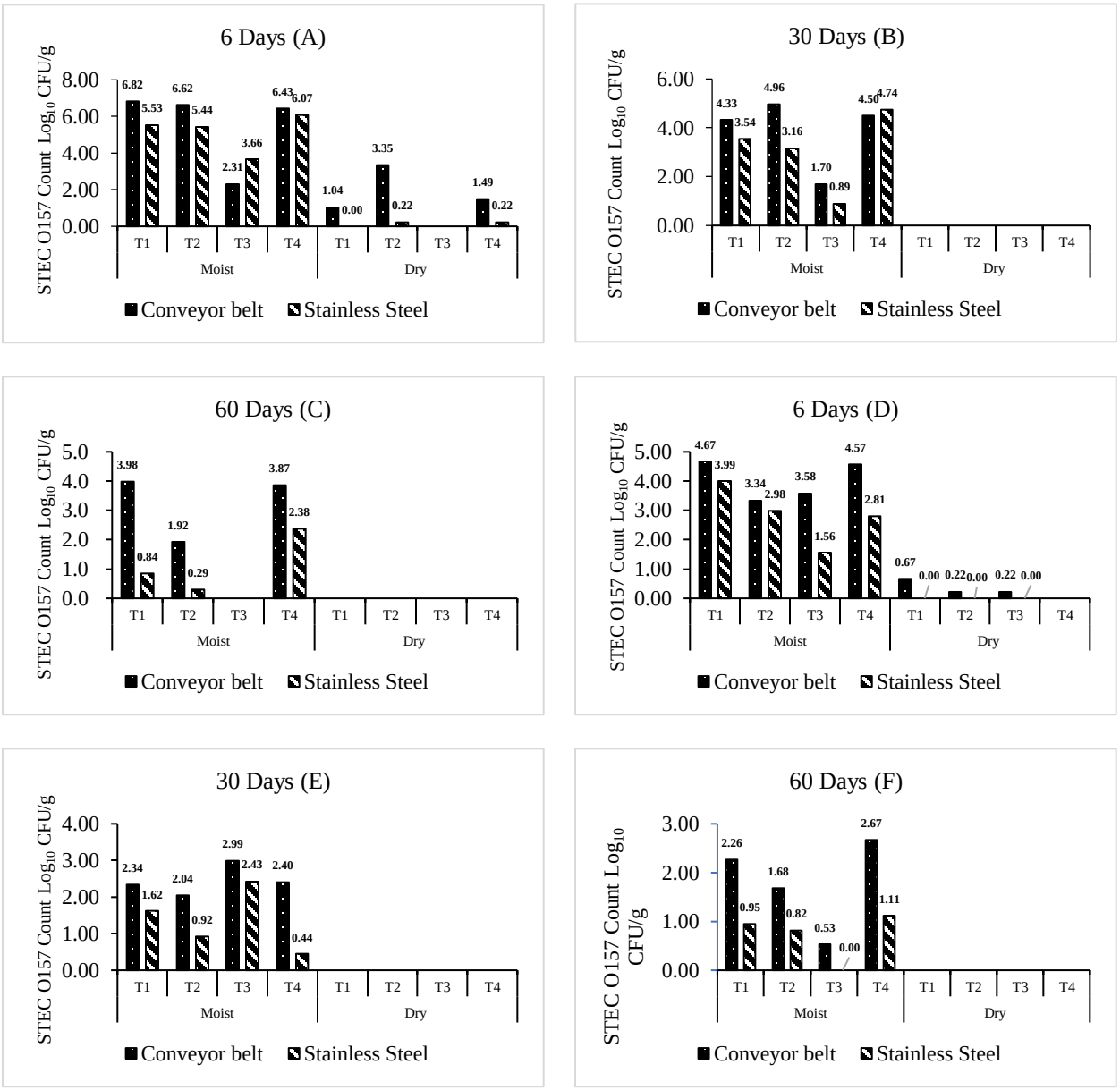

Figure 4-1. A, B, C; A number of O157:H7 cells transferred to beef from moist or dry multispecies biofilms formed at 25 °C for 6, 30, and 60 days. D, E, F; A number of O157:H7 cells transferred to beef from moist or dry multispecies biofilms formed at 10 °C for 6, 30, and 60 days. The four-strain combination were T1) *C. piscicola* + *L. bulgaricus*; T2) *C. koreensis* + *R. terrigena*; T3) *P. aeruginosa* + *C. koreensis*; and T4) STEC O157:H7 Control.

4.4.2. O157:H7 Survival Rate within Dry Biofilms

Within multispecies biofilms kept at 10 and 25 °C, it was found that STEC O157:H7 was in

sufficient numbers to be quantified at 6, 30, and 60 d. However, in dry biofilms at 30 and 60 d at both temperatures, STEC was only detectable by enrichment (Table 4-2).

At 25 °C, a synergistic effect was observed on biofilms formed on TPU, specifically by *C. koreensis* + *R. terrigena* (T2) and *C. piscicola* + *L. bulgaricus* (T1), which enhanced the O157:H7 survival rate from 0% (T4) to 67% (T2) and 22% (T1), respectively. Interestingly, these same biofilm combinations showed different survival rates on SS surfaces at 25 °C, where T2 decreased from 66.67% survival on TPU to 11.1% on SS, while T1 was reduced to 0% survival on SS surfaces. This indicated that the type of surfaces is impacting O157:H7 survival and transfer. Meanwhile, the 25 °C biofilm mixture *P. aeruginosa* + *C. koreensis* (T3) on TPU or SS surfaces showed no effect on O157:H7 survival rate, which was the same as control positive (0%).

At 10 °C, the O157:H7 recovery rate after enrichment on TPU was 88.9% for both T2 and T3 dry biofilm mixture, when compared with the control positive (0% recovery) indicating a synergistic effect. Temperature also shows a positive effect on O157:H7 survival in T3 and T2 mixed biofilms, since the observed recovery at 10 °C (88.9% and 88.9%) was higher than at 25 °C (0% and 66.6%, respectively). Also, interesting that no O157:H7 recovery (0%) was observed from T1 biofilm at 10 °C. (Table 4-2). Similar to 25 °C, 10 °C mixed biofilms showed a reduced survival rate on SS surface, where T2 had a 22% O157:H7 survival rate on SS, while no O157:H7 (0%) can be recovered from 10 °C T1, T3, and T4 multispecies biofilm on SS surface.

Table 4-2. Survival rate of STEC O157:H7 from dry multispecies biofilm after being stored at 25 °C for 60 days with and without a 24 h enrichment.

Surface	Strain combination	Recover without enrichment, % (n/N)	Recover with enrichment, % (n/N)	Total recover, % (n/N)
25 °C				
TPU	*C. piscicola* +*L. bulgaricus* (T1)	0.00 (0/9)	22.22 (2/9)	**22.22 (2/9)**
	C. koreensis + *R. terrigena* (T2)	0.00 (0/9)	66.67 (6/9)	**66.67 (6/9)**
	P. aeruginosa + *C. koreensis* (T3)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	O157:H7 Control positive (T4)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
SS	*C. piscicola* +*L.bulgaricus* (T1)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	C. koreensis + *R. terrigena* (T2)	0.00 (0/9)	11.11 (1/9)	**11.11 (1/9)**
	P. aeruginosa + *C. koreensis* (T3)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	O157:H7 Control positive (T4)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
10°C				
TPU	*C. piscicola* +*L.bulgaricus* (T1)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	C. koreensis + *R. terrigena* (T2)	0.00 (0/9)	88.89 (8/9)	**88.89 (8/9)**
	P. aeruginosa + *C. koreensis* (T3)	0.00 (0/9)	88.89 (8/9)	**88.89 (8/9)**
	O157:H7 Control positive (T4)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
SS	*C. piscicola* +*L.bulgaricus* (T1)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	C. koreensis + *R. terrigena* (T2)	0.00 (0/9)	22.22 (2/9)	**22.22 (2/9)**
	P. aeruginosa + *C. koreensis* (T3)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)
	O157:H7 Control positive (T4)	0.00 (0/9)	0.00 (0/9)	0.00 (0/9)

4.4.3. Scanning electron microscopy

Overall, the structures of 6-day biofilms developed by *C. koreensis* + *R. terrigena* (T2) and O157:H7 control positive (T4) at 25 °C (Figure 4-2) showed different thicknesses and cell density compared with their counterparts at 10 °C (Figure 4-3). At 25 °C, T2 and T4 biofilms displayed sporadically single-cell layers on the TPU and SS surface, with a few aggregates of rod-shaped bacteria. At 10 °C, *C. koreensis* + *R. terrigena* (T2) biofilms stored for 6 d showed a three-dimensional structure with abundant EPS matrix on the TPU and SS surface. In contrast, O157:H7 single-species biofilm (T4) was not observed on the TPU surface at 10 °C, and scattered clusters of bacteria in a monolayer were found on the SS surface.

At 25 °C, although a relatively larger cell aggregate and more EPS production T2 and T4 biofilm were observed in wet conditions than in dry biofilm, they were still shown as monolayer biofilm (Figure 4-2). On the contrary, the 10 °C dry multilayer biofilm *C. koreensis* + *R. terrigena* (T2) completely covered the TPU and SS surface, while wet multilayer biofilm T2 only partially covered the TPU and SS surface (Figure 4-3). However, a relatively larger cell aggregate and more EPS production by O157:H7 single-species biofilm (T4) were observed in 10 °C wet conditions instead of dry.

On the TPU surfaces (Figure 4-4), T2 (*C. koreensis* + *R. terrigena*) dry biofilms stored at 10 °C for 60 d, showed a multilayer structure. Meanwhile, *P. aeruginosa* + *C. koreensis* (T3) dry biofilm displayed as individual bacteria cells covered within EPS matrix and attached on the TPU surface.

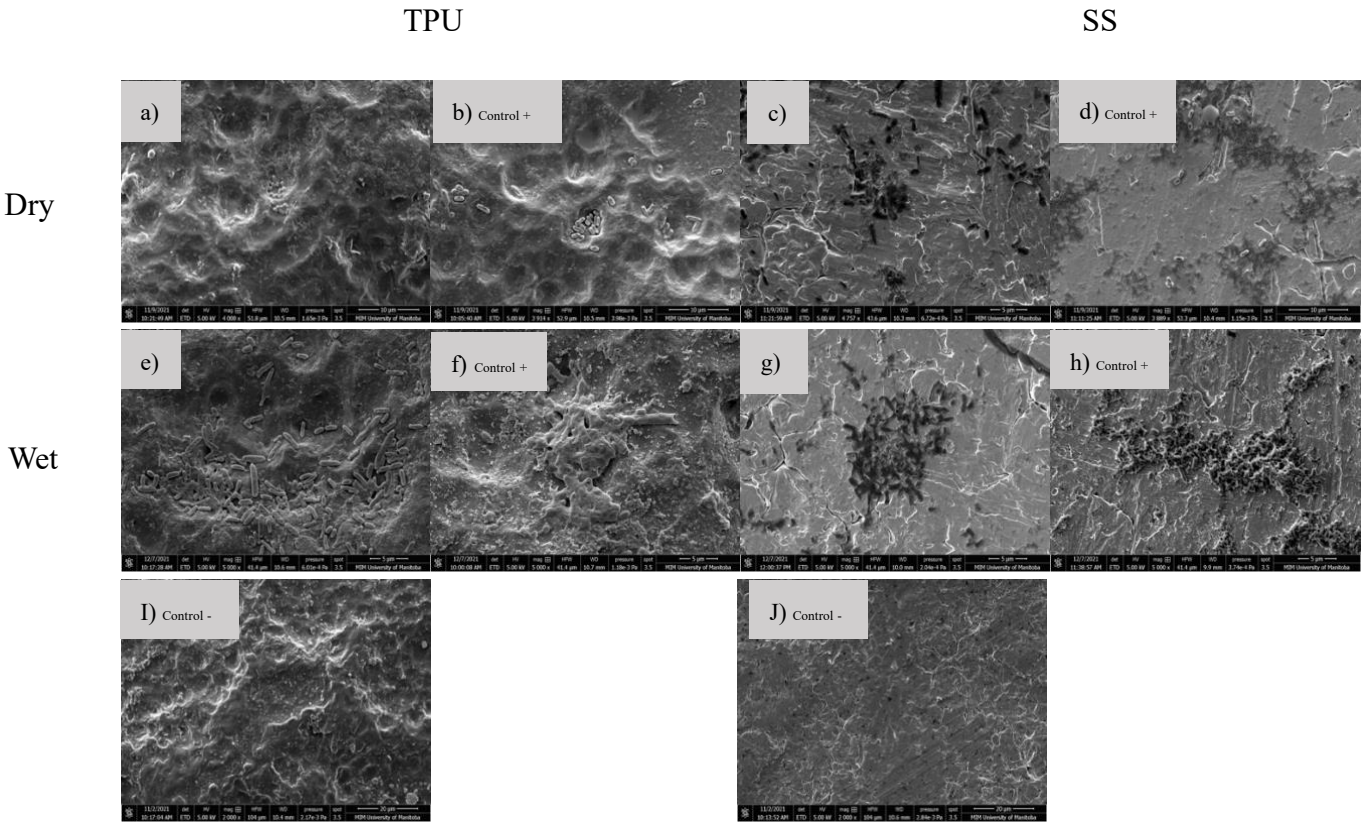

Figure 4-2. SEM of T2: *C. koreensis* + *R. terrigena* (a, c, e, and g) and T4: O157:H7 Control positive (b, d, f, and h) dry and wet biofilm stored at 25 °C for 6 days on a TPU and SS surface. SEM of I) TPU and J) SS coupon used as control negative, no bacteria observed.

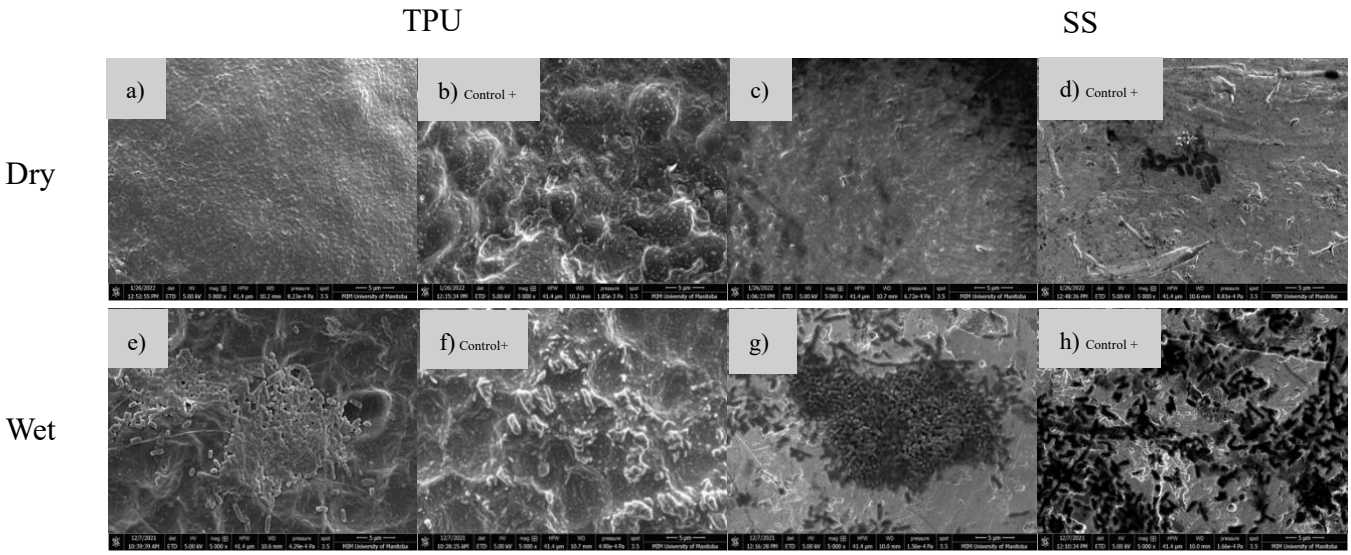

Figure 4-3. SEM of T2: *C. koreensis* + *R. terrigena* (a, c, e, and g) and T4: O157:H7 Control positive (b, d, f, and h) dry and wet biofilm stored at 10 °C for 6 days on a TPU and SS surface.

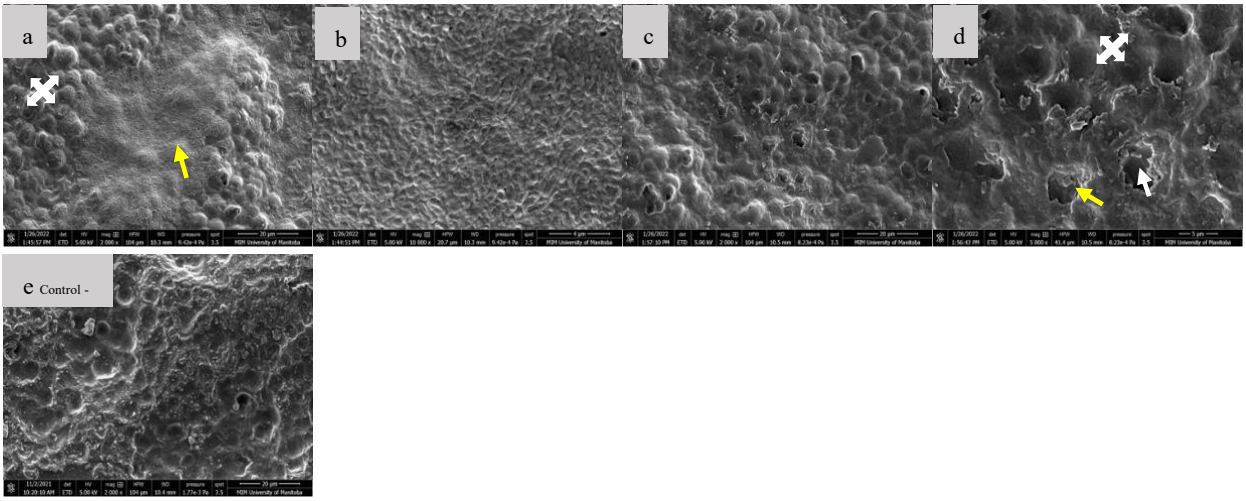

Figure 4-4. a and b) *C. koreensis* + *R. terrigena* (T2); and c and d) *P. aeruginosa* + *C. koreensis* (T3) dry biofilm stored at 10 °C for 60 days on a TPU are shown in different magnifications for better biofilm observation. SEM of e) TPU coupon used as control negative, no bacteria observed. Bacterial cells covered within extracellular polymeric substances (EPS) matrix (yellow arrow) was observed. Individual bacterial cells attached to coupon's surface and coated with EPS matrix (white arrows). And the white X indicates the TPU surface. On control negative e) irregular spherical sag structures are observed on the TPU surface, accompany with isolated particulate matter.

4.5. Discussion

4.5.1. Multispecies Biofilm Interaction with O157:H7

As reported elsewhere, the development of biofilms by foodborne pathogens on the top of pre-established multispecies biofilm on food contact surfaces is possible (Marouani-Gadri et al., 2009; Sofos and Geornaras, 2010). In the present study, *P. aeruginosa* + *C. koreensis* biofilm (T3) showed an antagonistic effect against O157:H7 at 25 °C, reducing O157 cells transfer to beef from 2.47 to 0.71 $\log_{10}$ CFU/g ($P < 0.001$) (Figure 4-1). Similar results were reported by Wang et al. (2013) on dual-species biofilms formed by STEC O157:H7 and *Salmonella typhimurium*. It was found that the participating species inoculated onto the surface first, showed to be the dominant member within mature dual-species biofilms (Wang et al., 2013b). The inhibition effect of *P. aeruginosa* + *C. koreensis* (T3) pre-established biofilm on O157:H7 development in a multispecies

biofilm is likely attributable to the competition for essential nutrients and attachment surface (Pang and Yuk, 2018; Wang, 2019). For example, both *Pseudomonas* sp. (Pang et al., 2017; Pang and Yuk, 2018) and *Comamonas* sp. (Carpentier and Chassaing, 2004) are capable of forming robust (thick) biofilms on stainless steel surfaces. In addition, *P. aeruginosa* can produce siderophores which can enhance its iron acquisition, which might deplete the iron from the surrounding environment and potentially limit the iron availability for O157:H7 (Cheng, 1995; Lopes et al., 2011; Rendueles and Ghigo, 2015). Moreover, the pre-existing biofilm of *C. piscicola* + *L. bulgaricus* (T1) and *C. koreensis* + *R. terrigena*. (T2) showed no effects on O157:H7 cell transfer to beef, which may indicate that the interaction between O157:H7 and pre-existed biofilm was dependent on participating bacterial species (Wang, 2019). For example, *P. aeruginosa* may prevent O157:H7 development in multispecies biofilm by releasing antimicrobials (Collazo et al., 2017; Kim et al., 2018). *P. aeruginosa* was demonstrated to produce harmful substances such as pigment pyocyanin, which are likely interfering with *E. coli* ability to thrive within the multispecies biofilm (Das and Das, 2015; Pang et al., 2017). Besides, other studies indicated that *P. aeruginosa* can produce signaling molecules cis-2-decanoic acid, which was shown to induce *E. coli* biofilm dispersion (Davies and Marques, 2009). Besides, *Pseudomonas* biofilm could produce enzymes activity and extracellular material differently under low-temperature conditions (Puga et al., 2016), which may explain why there is no synergistic/ antagonistic interaction between O157:H7 and *P. aeruginosa* at 10 °C.

4.5.2. Beef Contamination by O157:H7 and the Food Contact Surface type

Previous studies reported major cross-contamination events in the food processing facility are caused by bacteria transfer from the direct food contact surfaces (Pérez-Rodríguez et al., 2008; Wang, 2019). In the present study, the overall beef contamination with O157:H7 (1934) from TPU surfaces was significantly higher than from SS surfaces regardless of the temperature (Figure 4-1). Midelet et al. (2002) studied the extent of beef contamination by biofilm from different food contact surfaces. All the tested microorganisms, including *Listeria monocytogenes*,

Staphylococcus sciuri, *Pseudomonas putida*, or *Comamonas* sp., showed higher bacterial count and higher beef contamination on polyurethane than SS surface (Midelet and Carpentier, 2002). TPU causing higher bacterial contamination has been attributed to its higher hydrophobicity when compared to SS. Previous studies indicated that bacteria attach more rapidly on the more hydrophobic surface such as plastic (TPU) instead of stainless steel (Fletcher and Loeb, 1979; Pringle and Fletcher, 1983; Sinde and Carballo, 2000). In addition, several studies conducted in beef processing facilities have indicated that conveyor belts are commonly associated with the *E. coli* contamination on beef trimmings and cuts (Yang et al., 2015; Visvalingam et al., 2016; Yang et al., 2017a). Thus, O157:H7 planktonic cells present on the food contact surface could significantly be affected by the surface hydrophobicity, and the conveyor belt (TPU) might contribute to a higher likelihood of biofilm formation by O157 and beef contamination when compared with SS surfaces. In the present study, the O157:H7 recovery rate from 60 d dry biofilm on the TPU surface was higher than the SS surface (Table 4-2). Similarly, Adator et al. (2018) demonstrated that higher recovery rates of STECs showed on polystyrene plates than stainless steel 304 coupons. Therefore, the degree of beef contamination with STEC is likely to surface dependent and it should be considered when conducting risk assessments.

4.5.3. The Beef Contamination by O157:H7 Varies on Different Humidity and Storage Time

Previous studies indicated that one of the difficult challenges for proper sanitation the beef fabrication equipment is the meat debris removal, the meat debris persist on the equipment surface can protect bacteria against sanitization and nutrients for biofilm formation (Gill, 2009; Yang et al., 2017b). Multiple studies have shown that drying conditions can reduce the transfer of planktonic *E. coli* from meat debris on beef fabrication equipment to beef since gram-negative bacteria such as *E. coli* are generally sensitive to desiccation (Gill and Landers, 2004; Youssef et al., 2013). In the present study, storage of O157:H7 biofilm under a dry environment for 30 d can eliminate the transfer of viable O157:H7 cells to beef in both 10 and 25 °C (Figure 4-1). On the contrary, O157:H7 containing moist biofilm can continuously cause beef contamination for up to 60 d storage. However, the commercial sanitation protocol commonly applied by beef fabrication

plants includes washing the equipment with hot water (40 - 50 °C) for a few hours to remove soil, and the entire sanitation process can approximately take up to 9 hours (Wang et al., 2018). Thus, the routine sanitation process with hot water in a beef processing facility can easily maintain a high humidity condition in the plant (Møretrø et al., 2010), which could form condensation water and cause biofilm re-hydration. Meanwhile, the *E. coli* harbored in the meat debris, which persist on the beef fabrication equipment, could survive the sanitation process and likely form biofilm under the warm temperature (Gill, 2009; Visvalingam et al., 2016). Also, important to notice that biofilm formation is not just affecting the safety of the product, but also the quality, biofilms caused by spoilage bacteria or LAB are likely increasing bacterial loads on the beef product. Thus, if the beef fabrication equipment, including conveyor belts and stainless-steel surfaces, are regularly and efficiently cleaned, sanitized, and thoroughly dried, bacterial contamination during the beef fabrication may be avoided (Gill and Landers, 2004; Youssef et al., 2013).

4.5.4. *E. coli* O157:H7 Persistence in Dry Multispecies Biofilm and Long Storage Periods

With the long storage periods, the bacteria within the biofilm could experience osmotic stress, which might reduce the number of viable bacteria within the biofilm (Kim et al., 2008; Iibuchi et al., 2010). As expected, in this study an increased storage time was related to a reduced O157:H7 number transfer to beef. No O157:H7 transfer to beef was observed from 30 and 60 d dry biofilms (Figure 4-1). However, after enrichment, O157:H7 was recovered and varied according to the bacteria within the biofilm, humidity conditions, type of surface, and temperature. The recovery rate in *C. koreensis* + *R. terrigena* (T2) mixed dry biofilm was always higher than control positive (T4) in 60 d dry biofilm (Table 4-2). And biofilm mixture *C. piscicola* + *L. bulgaricus* (T1) and *P. aeruginosa* + *C. koreensis* (T3) were also showing synergistic effect enhancing O157:H7 recovery rate on TPU surface at 25 and 10 °C, respectively. Previous studies demonstrated that the EPS matrix can protect *Pseudomonas* sp. (Roberson and Firestone, 1992) and *Enterobacter* sp. (Kim et al., 2008) biofilm under desiccation conditions by retaining water and accumulating nutrients. EPS is secreted by the bacterial community and it can surround each bacteria cell, forming a complex

matrix (Flemming and Wingender, 2010). Puga et al. 2016, reported that the EPS such as polysaccharides produce by individual species could significantly influence multispecies biofilm development and its functional property (Puga et al., 2016). For example, in *E. coli* O157:H7 and *Salmonella typhimurium* dual-species biofilms, the presence of *Salmonella* was able to enhance *E. coli* O157:H7 and *Salmonella* resistance to sanitizers (Wang et al., 2013b). The author explained that the presence of *Salmonella* provides an additional EPS matrix in dual-species biofilm which might protect *E. coli* O157:H7 away from sanitizers (Wang et al., 2013b). In the present study, the multispecies dry biofilm T1 (*C. piscicola* + *L. bulgaricus*) and T2 (*C. koreensis* + *R. terrigena*) in 25°C, and T2 and T3 (*P. aeruginosa* + *C. koreensis*) in 10°C were surrounded by a thicker EPS matrix compared with the O157:H7 single species (T4) biofilm (Figure 4-2 4-4). Therefore, the additional EPS matrix produced by *C. koreensis* + *R. terrigena* (T2); *C. piscicola* + *L. bulgaricus* (T1); and *P. aeruginosa* + *C. koreensis* (T3) biofilm mixture could facilitate O157:H7 persistent in multispecies biofilm under desiccation condition. Thus, the multispecies biofilm developed on the beef fabrication equipment could continuously pose beef under the contamination risk (Skandamis et al., 2009). When these dry old biofilms become in contact with water or nutrients from beef products it could reactivate dormant cells and cause cross-contamination to spread and regrowth, as demonstrated in this research when enrichment was used to assess O157 survival (Table 4-2).

Results also showed that O157:H7 recovery varies with temperature. Multispecies biofilms *C. koreensis* + *R. terrigena* (T2) and *P. aeruginosa* + *C. koreensis* (T3) showed a higher O157:H7 recovery rate at 10 °C when compared with 25°C (Table 4-2). However, multispecies biofilm formed by *C. piscicola* + *L. bulgaricus* (T1) showed a better O157:H7 recovery rate at 25 °C than 10 °C. This likely indicated that the O157:H7 ability to persist in various temperatures depends also on the bacterial species that cohabit in the biofilm.

Other studies have investigated the ability of lactic acid bacteria (LAB) biofilms developed on the drainage systems in ready-to-eat poultry facilities, it was found that LAB biofilms can eliminate *Listeria* from floor drains (Zhao et al., 2004; Tong et al., 2013; Pérez Ibarreche et al., 2014). However, hypothetically LAB biofilm developed on beef fabrication equipment aiming to

reduce *Listeria* contamination, will likely interact with STEC transferred from the beef carcass during processing, which could enhance O157:H7 food contact and non-food contact colonization. In the food industry there is often more than one foodborne pathogen of concern, so the choice of biocontrol method using LAB or other bacteria or microorganisms to counteract specific foodborne pathogens needs to consider its efficacy regarding all the pathogens of concern. In the beef industry, the STEC bacteria attached to food contact surfaces are likely interacting with pre-established LAB multispecies biofilm. As a result, those STEC may persist within the LAB multispecies biofilm and likely particular LAB strains could alter these outcomes. Therefore, more studies should be performed to comprehensively evaluate the benefits and the hazards of applying different LAB strains in the food industry as a biocontrol method.

In the food industry, different technologies are used to control bacterial growth, one of the most common ones is temperature control. In the beef processing environment, the low environmental temperature (5-15 °C) likely limits enteric pathogens and spoilage bacteria from growing on food contact surfaces and the product (Ma et al., 2019; Ma et al., 2020). However, in the microbial world, not all bacteria behave the same when exposed to certain temperatures, or environmental conditions such as humidity variations or the presence of other bacteria. In the present study, when *E. coli* O157:H7 (1934) was tested in-vitro by using the crystal violate (CV) method, it was classified as weak-biofilm former at 10 °C (Table 4-1). However, further testing on TPU and SS surfaces showed that strain O157:H7 -1934 (T4) was capable to transfer cells from the biofilm to beef and was able to persist as a multispecies biofilm (Figure 4-1). These observations were confirmed with the SEM images (Figure 4-3). In the conventional sense, the food industry believed that those strong biofilm-forming pathogenic bacteria are more concerning than weak biofilm former by readily causing higher food cross-contamination (Keskinen et al., 2008). However, when strain 1934 was added to the pre-formed biofilm by *C. koreensis* + *R. terrigena* (T2), a robust biofilm was observed on TPU and SS surfaces at 10 °C and enhanced O157:H7 persistence during long period storage at 10 °C (Table 4-2). Likely the robust biofilm was formed primarily by *C. koreensis* + *R. terrigena* (T2) as previously observed when these

strains were tested individually, and O157 was able to later integrated within this biofilm, and benefit from it. This is an important finding since often spoilage bacteria are considered a food quality issue. Hence, findings in this research show that spoilage bacteria could also potentially affect food safety by enhancing foodborne pathogens' survival and persistence, and the biofilm-forming ability of pathogenic bacteria was not the sole determinant of the risk of food contamination.

4.6. Conclusion

This study demonstrates that SP and LAB bacteria commonly found in the food industry can affect *E. coli* O157:H7 persistence and survival. The biofilm mixture *P. aeruginosa* + *C. koreensis* (T3) was showed to be antagonistic towards O157:H7 at 25 °C. Interestingly, dry biofilm mixtures of *C. piscicola* + *L.bulgaricus* (T1), *C. koreensis* + *R. terrigena* (T2), and *P. aeruginosa* + *C. koreensis* (T3) enhanced O157:H7 survival rate under different temperatures after 60 d storage. The conditions for O157:H7 multispecies biofilm development, such as humidity condition, contact surface, and biofilm aging time, all have significant effects on biofilm formation, beef contamination, and O157 persistence. The overall beef contamination level with O157:H7 was higher when in contact with fresher (6 d) moist biofilm on conveyor belt surfaces. Therefore, further improvement in sanitation procedures to reduce biofilm formation on conveyor belts should be explored as a potential strategy to prevent beef contamination. Besides, O157:H7 can transfer to beef from moist biofilm even after 60 days, indicating the biofilm formation can continuously cause beef contamination. Although O157:H7 (strain 1934) is a weak biofilm former under 10 °C, the O157:H7 can enhance its desiccation resistance through integration within the pre-established multispecies biofilm. Hence, even weak biofilm former can pose a continuous risk to beef contamination.

Furthermore, we only investigated the spatial structure of O157:H7 multispecies biofilm by scanning electron microscopy technology. But evaluating the EPS chemical composition variation among different O157:H7 multispecies biofilm should be considered in future studies. The EPS

chemical analysis would provide additional information on the mechanisms of O157:H7 multispecies biofilm development and its persistence in the beef fabrication environment.

5. Chapter 5: Conclusion and Summary

Although the mechanisms of beef contamination with Shiga-toxigenic *Escherichia coli* (STEC) have been wildly studied, and multi-hurdle antimicrobial technique has been continuously implemented in the beef processing facilities (Gill, 2009; Yang et al., 2017a), the STEC are still associated with beef-related foodborne disease and outbreaks (CDC, 2016a; 2018; 2019c). Due to food safety concerns related to STEC contamination, significant economic losses can occur related to beef product recall and destruction (FSIS, 2014; Wang et al., 2016b). The establishment of biofilm on the beef fabrication equipment could act as a continuous source of beef contamination (Yang et al., 2015). Moreover, the STEC bacteria within biofilms are embedded within a self-produced extracellular polymeric substance, which enhances bacterial persistence and provides protection against selective pressure such as antimicrobial or desiccation (Srey et al., 2013; Vogeleer et al., 2014; Adator et al., 2018). In meat processing plants, biofilms are most often composed of multiple microorganisms (Wang, 2019). Biofilms may be composed of bacteria categorized as probiotics, spoilage, or pathogens (Fang et al., 2022), but little is known about the risk that these multispecies biofilms pose to beef. My study has demonstrated that multispecies biofilm *Raoultella terrigena* + *Comamonas koreensis* (T2) may provide a protection condition for STEC persistence under desiccation conditions. In contrast, multispecies biofilm *Pseudomonas aeruginosa* + *C. koreensis* (T3) can likely be developed as a bio-control method to prevent beef contamination with STEC. Although spoilage bacteria are not very desirable in food processing facilities, these findings are perhaps remaining us of the importance of understanding the impact of bacterial communities within food processing environments when developing biocontrol methods.

The primary objective of this study was to 1) evaluate potential synergistic and antagonistic interactions of STEC with either lactic acid bacteria (LAB) or spoilage bacteria (SP) within multispecies biofilms formed on thermoplastic polyurethane (TPU) or stainless steel (SS); 2) determine the extent of transfer of STEC from single and multispecies biofilms to beef with different storage times, surface, and humidity and 3) determine the capacity of STEC to survive

within single vs multispecies biofilms.

This work involved two STEC serogroups studies with different biofilm-forming abilities. In the first study, a strong biofilm producer STEC O103:H2 (99-2076) was tested, while an intermediate biofilm former STEC O157:H7 (1934) was selected in the second study. In both studies, the selected STEC serogroup's interactions with LAB or SP multispecies biofilms on TPU and SS coupons were evaluated at 10 °C and 25 °C under wet and dry conditions after 6, 30 & 60 d of storage. One LAB: T1 (*Carnobacterium piscicola* + *Lactobacillus bulgaricus*), and two SP: T2 (*Comamonas koreensis* + *Raoultella terrigena*); T3 (*Pseudomonas aeruginosa* + *C. koreensis*) were tested for their ability to form multispecies biofilms with STEC. The STEC single-species biofilms were treated as a control (T4). Coupons were stored under dry (20%-50% relative humidity; RH) or moist (60~90% RH) conditions after 6, 30 & 60 d of storage, and the capacity of STEC transfer to beef (2×2 cm^2) or survival was assessed.

Both studies demonstrated that STEC present in multispecies biofilm on food contact surface can transfer to the beef product after storage for up to 60 d. At 25 °C, multispecies biofilm *P. aeruginosa* + *C. koreensis* (T3) can decreased beef contamination with O103:H2 and O157:H7 by 2.54 $\log_{10}$ CFU/g ($P < 0.001$) and 1.76 $\log_{10}$ CFU/g ($P < 0.001$), respectively. These results were similar to those reported by Wang (2013) where a pre-established *Salmonella typhimurium* biofilm was able to outcompete *E. coli* O157:H7 in mature dual-species biofilms (Wang et al., 2013b). Another study showed that pre-formed *Pseudomonas veronii* biofilms were able to inhibit the growth of STEC O157:H7 on the SS coupons at 25 °C for 48 h (Kim et al., 2018), the author made the inference that O157:H7 growth inhibition could be attributed to *P. veronii* pre-formed biofilm initial advantage in nutrients and space competition (Alegre et al., 2013; Collazo et al., 2017). Thus, the antagonistic interaction between two STEC strains and pre-established *P. aeruginosa* + *C. koreensis* (T3) multispecies biofilm seems attributed to the competition of nutrients and attach surface (Alegre et al., 2013), and may further be investigated as biocontrol strategies (Collazo et al., 2017). Recently, the application of non-pathogenic bacteria on food, especially on the fruit and vegetable surface as a post-harvest control strategy has been widespread. For example, the

Pseudomonas syringae strain ESC-10 was commercialized as BioSave 10LP (Jet Harvest Solutions, Merritt Island, US) for fruit and potatoes mold prevention, and registered for use as fungicides in Canada (Health Canada, 2010). The *P. syringae* was previously demonstrated can prevent the growth of *E. coli* O157:H7 in apple wounds at 24 °C as a potential biocontrol method (Janisiewicz et al., 1999). However, the concern of developing multispecies biofilm on beef fabrication equipment is that the biofilms caused by spoilage bacteria or LAB are likely to increase bacterial loads on the beef product and promote meat deterioration. Other studies demonstrated that a rapid loss of melon color quality was observed when applying *Pseudomonas graminis* on melon cut surface to reduce *Salmonella* and *L. monocytogenes* growth (Abadias et al., 2014). Hence, the future study should further evaluate the impact of the selected multispecies biofilm on the organoleptic characteristic of beef products when developing biocontrol methods.

Interestingly, *C. piscicola* + *L. bulgaricus* (T1) and *C. koreensis* + *R. terrigena* (T2) mixed biofilms did not affect ($P > 0.05$) the extent of O103:H2 or O157:H7 beef contamination at 25 °C. Thus, the effect of multispecies biofilm on beef contamination with STEC seems to be dependent on the bacterial species. At 10 °C, none ($P > 0.05$) of the multispecies biofilms altered the transfer of O103:H2 or O157:H7 to beef compared to the control positive. Since the bacteria (e.g., *Pseudomonas*) enzyme activity and extracellular material production were different at low temperature (4°C) than 20°C (Puga et al., 2016), the antagonistic interaction between O103:H2 or O157:H7 with *P. aeruginosa* + *C. koreensis* (T3) multispecies biofilm was not observed at 10 °C. More research is needed to understand why STEC failed to establish itself into the *P. aeruginosa* + *C. koreensis* (T3) multispecies biofilm at 25°C instead of 10°C. Further research is needed to investigate the dynamic spatial distribution of the composed strain within the multispecies biofilm, which would necessitate confocal laser scanning microscopy (CLSM) accompanied by strain-specific fluorescent protein labeling techniques.

The present study demonstrated that environmental conditions such as contact surface type, humidity, temperature as well as biofilm aging, and presence of other bacterial species can affect the risk of beef contamination by STEC within multispecies biofilms developed on the food

contact surfaces. Biofilm developed on TPU surface can transfer higher ($P < 0.001$) STEC cell to beef sample than SS surface in both studies, which agrees with previous studies regarding the conveyor belt as the primary source that causes beef contamination with generic *E. coli* (Youssef et al., 2013; Yang et al., 2015; Yang et al., 2017b). In both studies, dry biofilm can significantly reduce ($P < 0.001$) STEC cell transfer to beef than moist biofilm, which indicated properly drying the beef fabrication equipment can protect the beef products from the risk of STEC contamination (Gill and Landers, 2004; Youssef et al., 2013). The STEC viable cell number transfer to beef was significantly reduced ($P < 0.001$) during 60 d storage in both studies. However, the moist biofilm can still cause beef contamination with STEC even after 60 d storage, while no beef contamination with STEC observed after contact with 60 d dry biofilm in both studies. In the beef industry, high humidity was common since routinely applying hot water (40 - 50 °C) in the sanitation process (Møretrø et al., 2010), the biofilm moist by condensation water on beef fabrication equipment can continually pose the beef under the risk of contamination.

Even though no STEC transfer to beef from 60 d dry biofilm was detected, the survival STEC can still be recovered after enrichment. Interestingly, the survival of O103:H2 was recovered from its dry single-species biofilm after 60 d storage; however, no O157:H7 survival was detected. One potential reason for those differences is that the O103:H2 (99-2076) was curli and cellulose producer, while the O157:H7 (1934) was lack of curli and cellulose producing ability. During biofilm development, curli fibers can associate with the cellulose to form a hydrophobic polymer (EPS) (Gualdi et al., 2008), and the EPS keeps the bacteria in a moist environment due to the high-water keeping capacity (Kumar and Anand, 1998a; Stewart and Franklin, 2008). Therefore, STEC strains able to express curli and cellulose are often correlated with a thicker biofilm formation and a higher persistence phenomenon (Gualdi et al., 2008; Iibuchi et al., 2010; Adator et al., 2018). Thus, the curli and cellulose producer (O103:H2) could better retard the biofilm dehydration and trap available nutrients within the biofilm than non-curli and cellulose producer (O157:H7) (Kumar and Anand, 1998a; Stewart and Franklin, 2008). Hence, the curli and cellulose-producing STEC are likely more persistent during the extended period of storage under the desiccated

condition.

Remarkably, the highest STEC recovery from 60 d *C. koreensis* + *R. terrigena* (T2) dry biofilm was observed in both studies at 10 °C, regardless of surface type. In Canada, beef processing facilities operate at temperatures below 10 °C to limit the growth of enteric pathogens (Visvalingam et al., 2017b; Yang et al., 2017a; Yang et al., 2017b). Both *C. koreensis* (25-64) and *R. terrigena* (ENT25-16) tested in the present studies were isolated from the conveyor belt in a beef fabrication facility previously (Wang et al., 2018). Therefore, those isolates should be adapted to forming biofilms at low temperature, and they readily form weak (*C. koreensis*) and strong (*R. terrigena*) biofilm at 10 °C in the present study. The previous study demonstrates that bacteria such as *C. koreensis* (25-64) and *R. terrigena* (ENT25-16) can survive the sanitation process and present on the conveyor belt (Wang et al., 2018), and the present study indicated that those bacteria can form multispecies biofilm and protect STEC bacteria which attached to the beef fabrication equipment against desiccation. As revealed by scanning electron microscopy, the multispecies biofilm *C. koreensis* + *R. terrigena* (T2) can form a 3-dimensional multilayer biofilm with extensively produced EPS matrix compared with O103:H2 and O157:H7 single-species biofilm. There is little information regarding the EPS component produced by *C. koreensis* and *R. terrigena* during biofilm formation under low temperatures such as 10 °C and how it interacts with STEC intergradation. Fang et al. (2022) demonstrated that *C. koreensis* (25-64) and *R. terrigena* (ENT25-16) could respectively produce curli-like substances and cellulose-like substances under 15 °C (Fang et al., 2022). Therefore, the robust biofilm formation and EPS (curli/cellulose-like substance) production of *C. koreensis* + *R. terrigena* (T2) at 10 °C likely covers the biofilm-like a gel to protect the STEC against desiccation (Kumar and Anand, 1998a; Stewart and Franklin, 2008). Further research was required to evaluate the STEC dynamic spatial distribution within the multispecies biofilm *C. koreensis* + *R. terrigena* (T2) by using CLSM accompanied by strain-specific fluorescent protein labeling. Meanwhile, the composition and distribution of *C. koreensis* + *R. terrigena* (T2) EPS matrix can also be investigated by CLSM technique, accompanied with attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and Raman

spectroscopy, to reveal the EPS chemical composition associated with STEC persistence ability within the dry multispecies biofilm.

Finally, this study demonstrated that O103:H2 and O157:H7 behave similarly on beef contamination when affected by different environmental conditions within the single or multispecies biofilm. However, O103:H2 and O157:H7 persistence ability under desiccation conditions differ in the different multispecies biofilm. For example, only *C. koreensis* + *R. terrigena* (T2) dry biofilm showed a synergistic effect on O103:H2 survival at 10 °C after 60 days of storage. In contrast, 60 d dry biofilm mixtures of *C. piscicola* + *L.bulgaricus* (T1), *C. koreensis* + *R. terrigena* (T2), and *Pseudomona* + *Comamonas* (T3) enhanced O157:H7 survival rate under different temperatures. Therefore, future studies should evaluate the general interaction between LAB or SP multispecies biofilm with top 7 STEC and should investigate the genetic makeup of STEC. Those studies are essential to comprehensively understand the impact of bacterial communities on the risk of beef contamination within the beef fabrication facility. Moreover, results demonstrated low temperature (10 °C) reduced O103:H2 and O157:H7 biofilm-forming ability and decreased STEC cell number transfer to beef. However, we should notice that the highest recovery rate for O103:H2 and O157:H7 has been observed from *C. koreensis* + *R. terrigena* (T2) biofilm at 10 °C in both studies. The finding from this study indicated that the beef industry should not evaluate the potential risk of beef contamination by STEC solely on its biofilm-forming ability, since STEC may persist on the beef fabrication equipment at low temperatures under the protection of bacterial communities and become a continuous source of chronic contamination.

In summary, the risk of beef contamination with STEC relies on the species composition, attachment surface, humidity, and the biofilms age. The multispecies biofilm developed on the beef fabrication equipment could persistently pose beef under the contamination risk. Especially when these old dry biofilms moistened by water or nutrients from beef products (Gill et al., 1999), it could resume regrowth and cause cross-contamination, as observed in this research when enrichment was used to assess O103:H2 and O157:H7 survival. These findings advance current

knowledge on the ecology of multispecies biofilms and the factors that influence their development within beef processing facilities. Besides, the implementation of Hazard Analysis and Critical Control Points (HACCP) system in beef processing facility integrated with multiple sanitation procedures as hurdle technology to prevent the biofilm formation on the conveyor belt is essential.

5.1. Prospects

The mechanisms of STEC containing multispecies biofilm formation and persistence are complex and can continuously pose a challenge to beef producers. In the presented research much research was done, however, more questions were raised. Further investigation is needed. I think that among the next step will be to 1) investigate the dynamic spatial distribution of the composed strain within the multispecies biofilm; 2) to determine the EPS chemical composition associated with STEC persistence ability within the dry multispecies biofilm; 3) to evaluate the general interaction between LAB or SP multispecies biofilm with top 7 STEC and should look into the genetic makeup of STEC. By performing those further studies, we can more comprehensively understand whether the spatial distribution of STEC within the multispecies biofilm can contribute to its persistence in desiccation conditions. In addition, further studies can determine whether it is feasible to select the appropriate protease or polysaccharase for biofilm EPS degradation and biofilm removal; and whether the beef contamination risks associated with multispecies biofilms containing other STEC serotypes can be predicted.

In the present study, SEM imaging was applied to verify the biofilm establishment on the food contact surface. However, SEM technique cannot reveal the dynamic inter-species interaction during the multispecies biofilm development (Wang et al., 2013b). Therefore, in order to study the STEC spatial distribution within multispecies biofilm formation and persistence in a real-time way, confocal laser scanning microscopy (CLSM) accompanied by strain-specific fluorescent protein labeling techniques was required (Chen et al., 2015). Meanwhile, we can also determine the spatial and temporal distribution of STEC cells within multispecies biofilm during long-term storage in a non-destructive way (Chen et al., 2015).

Additionally, the STEC persistence in multispecies biofilm during long-period storage in my study was attributed to the complex EPS matrix produced by *C. koreensis* + *R. terrigena* (T2) during biofilm formation. Currently, each multispecies biofilm mixture's specific EPS matrix composition remains unknown since the SEM imaging cannot be utilized to investigate the detailed EPS matrix distribution, including protein, polysaccharide, and eDNA, within the multispecies biofilm. Thus, the CLSM technique could be applied with attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and Raman spectroscopy to reveal the EPS chemical composition associated with STEC persistence ability within the dry multispecies biofilm. Understanding STEC spatial distribution and detailed EPS chemical composition during biofilm development may provide enough knowledge for multispecies biofilms removal strategy development. For example, EPS disruption by pre-selected polysaccharides and proteinase can decrease biofilm formation and further compromise STEC persistence (Vogeleer et al., 2014; Kim et al., 2021). Meanwhile, vibrational techniques such as Near-infrared spectroscopy (NIRS) can facilitate biofilm detection by using the chemical components related to STEC persistence as predictors.

This research showed that the beef contamination risk with STEC varied with the biofilm species composition, and the presence of *C. koreensis* + *R. terrigena* (T2) can enhanced the STEC survival and persistence. However, further questions remain regarding other “top seven” serotypes, we only tested O103:H2 and O157:H7 and some spoilage microorganism, however, each environment is unique, and we don’t have enough information to predict risk associated with other STEC serotypes or different spoilage communities. Multiple previous studies indicate that different STEC serotypes showed varied stress responses, which is likely attributed to their genetic and metabolic diversity (Chen et al., 2020; Wang et al., 2022; Zhao et al., 2022). Hence, different STEC serogroups could vary the interspecies interaction in a multispecies biofilm. Multiple studies indicated that the genic profile alone cannot accurately reveal the biofilm formation mechanisms in STEC, since the gene expression regulation in biofilm formation was impacted by environment condition and accompany bacteria species (Kim et al., 2009; Landini, 2009; Fang et al., 2022).

Therefore, future studies should evaluate the general interaction between LAB or SP multispecies biofilm with the rest “top seven” STEC and accompany with the transcriptomic analysis of STEC. Those studies are essential to comprehensively understand the impact of bacterial communities on the risk of beef contamination within the beef fabrication facility.

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7. Appendix

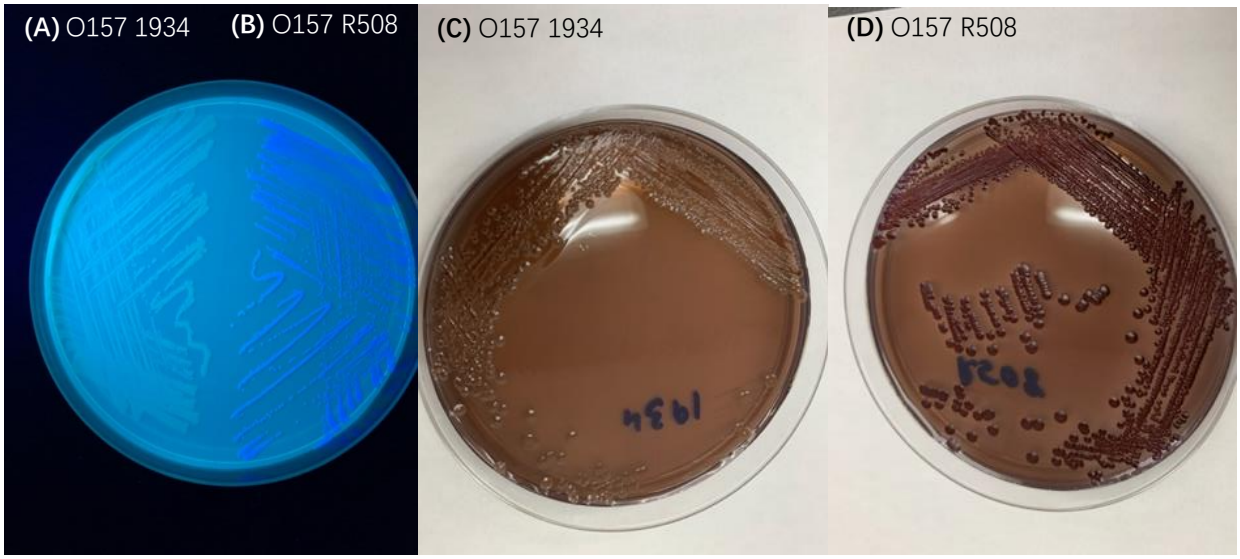

Figure 7-1. Cellulose and curli expression of STEC on LB agar (366-nm UV light) and CRI agar, respectively. The colony phenotype showing **(A)** cellulose negative, **(B)** cellulose positive, **(C)** curli negative, and **(D)** curli positive.

Table 7-1. Curli and cellulose production of the STEC and generic E. coli strains at 37 °C.

Strain	Cellulose	Curli
O26: H11 (00-3941)	-	+
O45: H7 (05-6545)	-	-
O103: H2 (99-2076)	-	+
O111: NM (CFS3)	-	+
O121: H19 (03-2832)	-	+
O145: H2 (75-83)	-	+
O157: H7 (1934)	-	+
O157: H7 (1931)	-	-
O157: H7 (R508)	+	+
Generic *E. coli* (8_77)	+	+
Generic *E. coli* (7_16)	-	-