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ORIGINAL ARTICLE

Lactic acid bacteria and spoilage bacteria: Their interactions in *Escherichia coli* O157:H7 biofilms on food contact surfaces and implications for beef contamination

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Abstract

This research explores the interaction between Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and bacteria species commonly found in beef processing environments, specifically *Carnobacterium*, *Lactobacillus*, *Comamonas*, *Raoultella*, and *Pseudomonas*. The study investigated how various environmental conditions impact the formation of biofilms and the ability of O157:H7 to transfer from multispecies biofilm onto beef surfaces. For this purpose, a mixture of lactic acid bacteria (LAB), spoilage bacteria (10^6 CFU/mL), and *E. coli* O157 (10^3 CFU/mL) were combined as follows: LAB (**T1**): *Carnobacterium piscicola* + *Lactobacillus bulgaricus* + O157:H7, an spoilage bacteria (**T2**): *Comamonas koreensis* + *Raoultella terrigena* + *O157:H7*, an spoilage bacteria (**T3**): *Pseudomonas aeruginosa* + *C. koreensis* strain + O157:H7 and only O157:H7 as control (**T4**). Multispecies biofilms were developed on thermoplastic polyurethane (TPU) and stainless steel (SS) coupons at 10 and 25°C for 6 days, washed and stored for 6, 30, and 60 days at wet (60%–90% RH) and dry (20%–50%, RH) conditions. To evaluate O157:H7 transfer, beef cubes (3 × 3 × 1 cm) were placed on the coupons, followed by a 50-g weight (7.35 kPa). The experiment was repeated three times in triplicate for each strain combination. Results demonstrate that biofilms formed at 10°C were generally weaker (less biomass) than those at 25°C. Regardless of temperature, more viable O157:H7 cells were transferred to beef from moist biofilms on TPU surfaces. At 25°C, T3 biofilm exhibited the lowest O157:H7 transfer to beef by 1.44 $\log_{10}$ CFU/cm^2 ($p < 0.01$). At 10°C, none of the multispecies biofilm (T1–T3) affected the number of O157:H7 transfers to beef ($p > 0.05$). Notably, O157:H7 was not detected on food contact surfaces with 30 and 60-day-old dry biofilms (T1–T4). Through enrichment, *E. coli* O157:H7 was recovered from multispecies biofilms T1, T2, and T3. Findings from this study imply that multispecies biofilms contribute to the persistence of O157:H7 under dry conditions, regardless of temperature. These results underscore the intricate influence of multiple environmental factors—including surface type, biofilm age, humidity, temperature,

and the presence of other bacterial species—on the risk of beef contamination facilitated by biofilms.

KEYWORDS
beef, biofilm, contamination, multispecies, persistence, STEC

1 | INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are globally significant enteric pathogens (CDC, 2014; Nguyen & Sperandio, 2012). STEC strains possess various virulent factors that contribute to their pathogenicity. Among these virulence factors, *eae*, *ehxA/hlyA*, stx_1, and stx_2 play significant roles in the development of STEC-related illnesses (Deng et al., 2004; Padola & Etcheverria, 2014). While stx_1 and stx_2 are implicated in disease pathogenesis, stx_2 has been consistently associated with more severe outcomes, including an increased risk of Hemolytic Uremic Syndrome (HUS) (Kume et al., 2022). Even the consumption of a small number of STEC cells, as few as 10, can result in severe complications such as hemorrhagic colitis and HUS (Etcheverria et al., 2010; PHAC, 2015). In 2019, the Canadian National Enteric Surveillance Program (NESP) reported 1462 cases of STEC infections, with only 27% attributed to O157 STEC (PHAC, 2020). However, in 2018, O157:H7 was still the most common STEC serotype isolated from clinical cases (34.48%), followed by O26:H11 (11.49%), O111:NM (5.75%), and O103:H2 (5.75%) (PHAC, 2019). The highly pathogenic nature of O157:H7 makes it a significant food safety concern in North America (Noftall et al., 2019).

Ruminants, particularly cattle, are considered the primary reservoir of STEC (Bryan et al., 2015). Hides and feces of ruminants are frequently identified as sources of contamination for carcasses, beef primals, and sub-primals (Bryan et al., 2015; PHAC, 2015). Within beef processing facilities, testing of beef trims has revealed periods of exceptionally high risk for STEC contamination, known as high event periods (HEP). Although the causes of HEP are unknown (Stanford et al., 2017), recent studies have suggested that biofilms play a role in this phenomenon (Wang et al., 2014, 2016).

Biofilms are single-species or multispecies bacterial communities that attach to surfaces and are embedded within a self-produced extracellular polymeric matrix (EPM) (Adator et al., 2018; Srey et al., 2013; Vogeleer et al., 2014). Biofilms provide a protective environment for bacteria, enhancing their ability to withstand environmental stresses and facilitating their long-term survival (Marouani-Gadri et al., 2009). Furthermore, biofilms can serve as reservoirs for bacterial transfer to food. Arthur et al. (2014) collected a total of 639 beef trim samples that were associated with 21 HEP and noted that each individual HEP was dominated by a single O157:H7 strain type, suggesting a common origin (Arthur et al., 2014). *E. coli* O157:H7 strains isolated from HEP can readily formed biofilms and have been shown resistant to a number of sanitizers (Wang et al., 2014). In a study by Stanford et al. (2021), STEC originated from cattle and beef processing environments in western Canada were tested to determine biofilm formation potential at 15°C using the crystal violet method. Results showed that out of 745 STEC isolates from cattle only a single STEC strain exhibited strong biofilm-forming ability (Stanford et al., 2021). The authors claim that even these few strong biofilm formers carried by cattle could accumulate within the meat processing environment and potentially contributing to HEP (Stanford et al., 2021). In the beef processing facilities temperature variations occur during fabrication, thus a limitation of the study by Stanford et al. (2021), is that the STEC strains were only tested at 15°C, which does not reflect the ability of these strains to form biofilm at other temperatures. Adator et al. (2018) developed STEC biofilms stainless steel at 25°C under wet conditions and then let it dry (40%–50% relative humidity) for 30 days. Biofilms were tested to determine STEC viability, and it was found that the top seven STEC strains retained their viability within biofilms on dry surfaces for a minimum of 30 days. These strains could transfer to lettuce within a short span of 2 min upon exposure.

Within food processing facilities, biofilms often consist of multiple bacterial species (Marouani-Gadri et al., 2009), and STEC persistence could be enhanced by background microbiota through multispecies biofilms. Some of the bacteria often associated with beef products are *Pseudomona*, *Commamonas*, and *Lactobacillus*, including *Carnobacterium* and *Raoultella* (Borch et al., 1996; Caturla et al., 2023; Ercolini et al., 2006). Bacteria carried by raw material also become part of the final product microflora and part of the food processing environment microbiome. In beef processing facilities, both lactic acid bacteria (LAB, e.g., *Carnobacterium* sp.) and spoilage bacteria (SP, e.g., *Raoultella* sp.) have been found to persist on beef fabrication equipment such as conveyor belts (Wang et al., 2018). During beef processing, it is possible that STEC attached to the food contact surface could integrate into pre-established multispecies biofilm (Giaouris et al., 2015; Wang, 2019). However, our current knowledge about the role of pre-established multispecies biofilms in food processing facilities is limited. To develop practical risk assessment tools and pathogen reduction interventions, it is crucial to investigate the interactions between pathogenic bacteria like O157:H7 and other bacterial species (e.g., LAB) that can coexist within biofilms in food processing environments (Chitlapilly Dass et al., 2020; Giaouris et al., 2015). A better understanding of such interactions can help assess the potential risks associated with the presence of certain bacteria and the persistence of foodborne pathogens, possibly laying the groundwork for shaping upcoming research inquiries involving the food processing microbiome on the sustained presence of foodborne pathogens. Further research on the variables that affect STEC resilience will also aid in the development of improved pathogen biocontrol strategies for

TABLE 1 Shiga Toxigenic *Escherichia* coli, lactic acid bacteria and spoilage bacteria selected for multispecies biofilm development using 96-microplate well crystal violet technique.

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

[a]O157:H7 1934 carry genes for *stx*1, *stx*2, *eae* & *hly*A.
[b]Biofilm-forming ability is as follows: non (OD < ODc), weak (ODc < OD < 2ODc), intermediate (2ODc < OD < 4ODc), and strong (4ODc < OD) biofilm formers.
Source: Chekabab et al. (2013), Nan et al. (2022).

the food industry (Burmolle et al., 2014; Rendueles & Ghigo, 2015). Thus, the primary goal of this research was to explore the interaction of *E. coli* O157:H7 with selected bacterial species isolated from beef plants or beef products. Additionally, we aimed to investigate the influence of various environmental variables on STEC biofilm formation. In this study, we examined the capacity of O157:H7 (strain 1934) to establish multispecies biofilms on food contact surfaces and contaminate beef using the following three strain combinations: one LAB combination, **T1**: *Carnobacterium piscicola* + *Lactobacillus bulgaricus*, and two spoilage bacteria combinations, **T2**: *Comamonas koreensis* + *Raoultella terrigena*, and **T3**: *Pseudomonas aeruginosa* + *C. koreensis*. The selection of these strains and their combinations was based on results from a previous study conducted by Nan et al. (2022). Briefly, three strain combinations were selected from 12 SP and 12 LAB bacteria; the selection was based on their ability to form biofilms individually and by their interactions with *E. coli* O103:H2 (Nan et al., 2022). Since LAB bacteria are typically associated with spoilage (Hernández-Macedo et al., 2011), in the scope of this study, we refer to LAB as a separate group due to their potential to be used as an intervention to mitigate surface contamination by pathogens in food processing environments (Cisneros et al., 2021).

The objectives of this study were: (1) assess potential synergistic or antagonistic effects of selected LAB or SP multispecies biofilms with O157:H7 on thermoplastic polyurethane (TPU) and stainless steel (SS) surfaces, considering different storage times, temperatures, and humidity levels; (2) determine the extent of O157:H7 transfer from single and multispecies biofilms to beef under these conditions; and (3) evaluate the survival rate of O157:H7 within single and multispecies biofilms.

2 | METHODS AND MATERIALS

2.1 | Bacteria strains and culture conditions

Based on previous work by our group, one STEC O157:H7 strain, three SP (*Comammonas*, *Raoultella*, and *Pseudomonas*) and two LAB strains (*Carnobacterium* and *Lactobacillus*) were selected to be tested in this study (Table 1). Selected bacteria showed intermediate to strong biofilm-forming ability at either 10 or 25°C. The choice of the O157:H7 1934 strain was guided by its distinct characteristics. This strain is categorized as intermediate biofilm former, and it lacks curli and cellulose-producing capabilities (phenotypically), as outlined in Table 1. For further insight into the selection criteria applied to the bacterial combinations included in this study, details can be found in Nan et al. (2022). The 96-well microplates crystal violet method was used to assess bacterial strain's ability to form biofilms as described elsewhere (Nan et al., 2022; Wang et al., 2016). The criteria to designate biofilm formation ability was based on the optical density cutoff (ODc), which was determined as the sum of three times the standard deviation of the negative control's readings and the mean value of the negative control. Subsequently, each strain's biofilm-forming proficiency was categorized into four groups: non-biofilm former (OD < ODc), weak biofilm former (ODc < OD < 2ODc), intermediate biofilm former (2ODc < OD < 4ODc), and strong biofilm former (4ODc < OD) (Adator et al., 2018). The STEC O157:H7 1934 was shown to be a weak and intermediate biofilm former at 10 or 25°C, respectively (Table 1).

All bacterial strains were stored at −80°C in Lennox 15% glycerol broth (LB-NS; Tryptone 10 g/L and Yeast extract 5 g/L) without NaCl. The SP and LAB strains were cultured on Trypticase Soy Agar (TSA; Becton and Dickinson, Sparks, USA), while the O157:H7 strain was cultured on MacConkey agar plates (Hardy Diagnostics Inc., Santa Maria, USA) at 25°C. This temperature was selected following initial tests aimed at determining an optimal growth temperature for all the strains that were examined. One individual colony from each culture was picked from each agar plate, transferred into 10 mL LB-NS, and grown to a density corresponding to 10^8 CFU/mL.

To mimic beef fabrication environments, a sterile beef purge was added to LB-NS broth (Pang & Yuk, 2018). Several packages of vacuum-packed beef (i.e., eye of round with a fat cap) were purchased to prepare the beef purge. All the beef purge was collected at the same time and diluted 1:6 with distilled water. The diluted beef purge was then filter-sterilized through a 0.45 μm filter (Midelet & Carpentier, 2002), and its protein content was measured using a Coomassie (Bradford) protein assay kit (Thermo Scientific, Rockford, USA)

(Kruger, 2009). The diluted sterile beef purge (14.3%) stock was stored in separate vials at −20°C, with each vial thawed and added into LB-NS broth (10% v/v; mLB-NS) as needed.

2.2 | Culture combination

Three strain-combinations were used to form multispecies biofilms with O157:H7 (strain 1934) on food contact surfaces, which included: one LAB combination, **T1**: *Carnobacterium piscicola* + *Lactobacillus bulgaricus*, and two spoilage bacteria combinations **T2**: *Comamonas koreensis* + *Raoultella terrigena*, and **T3**: *Pseudomonas aeruginosa* + *C. koreensis*. In addition, *E. coli* O157:H7 (strain 1934) single-species biofilms were developed as a positive control (**T4**). Bacterial biofilms were formed as per Wang et al. (2013) with minor modifications. Overnight cultures of each LAB and SP strain were diluted to 10^6 CFU/mL in mLB-NS and combined to form mixed cultures according to the experimental design (Wang et al., 2013).

2.3 | Polyurethane and stainless-steel coupon preparation

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

Stainless steel 304 coupons (SS) (2 cm-diameter; Pegen Industries Inc., Stittsville, Canada) were washed with distilled water and sonicated in an ultrasonic water bath for 20 min at 60°C before and after sonication in aqueous phosphoric acid (15% v/v) for 20 min at 60°C (Adator et al., 2018). Coupons were then dry sterilized in an autoclave before use. Bacterial enumeration was performed for randomly selected three TPU and SS coupons from each batch.

2.4 | Dry and wet multispecies biofilm formation

Coupons were placed into sterile Petri dishes (60 × 15 mm; VWR™, Radnor, USA), and 5 mL of either LAB or SP bacterial cultures (10^6 CFU/mL) was transferred onto each coupon (Adator et al., 2018). Coupons were stored at either 10 or 25°C for 6 days to allow biofilms to form. On day 6, coupons were washed three times (10 mL/coupon) with Butterfield's Phosphate Buffer (BPB) and transferred to a new sterile Petri dish. Then, aliquots (5 mL) of O157:H7 (10^3 CFU/mL) were added to the preformed biofilms on coupons and plates were incubated for another 6 days at either 10 or 25°C. Single-species O157:H7 biofilms were also developed as a positive control (**T4**); coupons which received no inoculant served as a negative control. Coupons were washed as previously described and dried for 4 h at room temperature in a Biosafety cabinet. Three repetitions by triplicate ($n = 9$) were conducted. Coupons were stored in sealed plastic containers along with a digital thermometer plus humidity meter, under either moist (60%–90% relative humidity, (RH)) or dry (20%–50% RH) conditions at the two respective temperatures. Moist biofilms were sprayed with sterile water (150 µL/coupons) once daily. A subgroup of SS and TPU coupons with biofilms were assessed for their capacity to transfer O157:H7 to beef, and a companion set was used to enumerate O157:H7 within biofilms after 6, 30, and 60 days.

2.5 | Beef samples preparation to test O157:H7 transfer

Vacuum-packed eye of round beef whole cuts (with a fat cap) were purchased, stored at 4°C and used within one week. The meat surface was washed and soaked in a 5% lactic acid solution for 1 min. to reduce background flora (Youssef et al., 2013). After immersion in lactic acid, each cut was drained for 1 min, trimmed off the outer surface layer, and then sectioned into 3 × 3 cm pieces using a sterile technique. Diced meat was stored at 4°C for less than 24 h prior to use. The presence of naturally occurring *E. coli* on the diced beef was evaluated by selecting three beef pieces, which were placed in individual Whirl-Pak bags (Nasco®; Madison, USA) containing 9 mL of Buffered peptone water (BPW; Hardy Diagnostics Inc., Santa Maria, USA). Bags were then homogenized using a stomacher for 1 min (Intersciences Inc., Markham, Canada) before 100 µL was plated onto MacConkey agar.

2.6 | *E. coli* O157:H7 transfer from biofilms to beef and O157:H7 biofilm enumeration

To assess O157:H7 transfer from biofilms, beef pieces (3 × 3 × 1 cm) were placed on top of TPU or SS coupons. A 50 g weight was then placed on top of the beef piece to exert 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. After 5 min of contact, beef pieces were removed from each coupon, placed into Whirl-Pak bags containing 9 mL of BPW, and homogenized in a stomacher for 1 min. To enumerate O157:H7, 10-fold dilutions were performed and plated on TSA overlayed MacConkey agar (Wu, 2008). The TSA overlay enabled potentially stressed O157:H7 cells to recover (Medina et al., 2020). Plates were incubated at 37°C for 24 h. Presumptive *E. coli* colonies were confirmed as STEC O157 by serological (O-antigens) agglutination (SSI Diagnostica, Hillerod, Denmark) and PCR (Adator et al., 2018).

Samples of beef pieces that did not result in STEC colonies on MacConkey plates (undetectable levels <10 CFU/cm^2) were enriched in modified tryptone soya broth (mTSB; Oxoid Ltd., Nepean, Canada) for an additional 24 h at 37°C, followed by spreading of 100 µL onto MacConkey agar and confirmed by latex agglutination and/or PCR (DebRoy et al., 2011).

To quantify O157:H7 on TPU and SS coupons, these were transferred into Whirl-Pak bags along with 9 mL of BPW to achieve a 10-fold dilution and then sonicated for 1 min (Marouani-Gadri et al., 2009). For O157:H7 enumeration, the drop plate method was used with five drops (10 µL/drop) dispensed on each plate (Herigstad et al., 2001). TPU and SS coupons that did not result in STEC colonies on MacConkey plates (<10 CFU/cm^2) after 24 h at 37°C were enriched, and isolates were confirmed as described above.

2.7 | Scanning electron microscopy

The mature multispecies biofilms of *C. koreensis* + *R. terrigena* (T2) and *P. aeruginosa* + *C. koreensis* (T3) with O157:H7 on TPU and SS coupons were visualized using scanning electron microscopy (SEM) at the Manitoba Institute for Materials (MIM). The TPU and SS coupons were fixed in 10% buffered formalin (Sigma Aldrich, St. Louis, USA) for 2 h and then washed and immersed in BPB for 30 min (Adator et al., 2018). Coupons were air-dried for 4 h at room temperature, and the TPU surfaces were coated with Gold–Palladium (Denton Vacuum Desk II, Moorestown, USA). Biofilms were then visualized using a Quanta™ 650 FEG scanning electron microscope (FEI CO., Hillsboro, USA) at 5 KV in the high-vacuum mode.

2.8 | Multispecies biofilm biomass detection and bacteria enumeration

To assess the EPM contribution of each strain to the multispecies biofilms, the biofilm mass was determined by individual strains and in combination. Single and multispecies biofilms were developed as previously described (Nan et al., 2022). Briefly, three multispecies biofilms included: *C. piscicola* + *L. bulgaricus* (T1), *C. koreensis* + *R. terrigena* (T2), and *P. aeruginosa* + *C. koreensis* (T3) were pre-established in microplate after 6 days' incubation at 10 and 25°C. Then, O157:H7 (strain 1934) was inoculated (10^3 CFU/mL) using sterile serological pipettes into each pre-established multispecies biofilm to develop mature biofilms for an additional 6 days of incubation. *E. coli* O157:H7 single-species biofilms were included as positive controls (**T4**).

To assess the biomass (EPM) production, mature multispecies biofilms were stained with 0.1% Crystal Violet (CV) as previously described by Nan et al. (2022). Microplates were washed with 300 µL of BPB per well three times by using a microplate washer (405 LS; BioTek, Winooski, USA). The washed microplates were air-dried, and the biofilm was fixed with 200 µL methanol, followed by 200 µL CV staining for 15 min. After washing three times with 300 µL of BPB, the residual CV in the biofilms was solubilized by adding 200 µL ethanol (85% v/v). The biomass-producing ability was indirectly evaluated by detecting residual CV at 630 nm using a microplate reader (BioTek ELx800; BioTek Instruments Inc., Winooski, USA). Three repetitions were performed for each strain combination, with a total of 48 wells per strain combination.

To verify the multispecies biofilm formation on the food contact surface, the participating LAB or SP bacteria were differentially enumerated after *E. coli* O157:H7 (strain 1934) integrated into the pre-established biofilm and developed multispecies biofilm on the food contact surface. Three multispecies biofilms, T1 (*C. piscicola* + *L. bulgaricus*), T2 (*C. koreensis* + *R. terrigena*), and T3 (*P. aeruginosa* + *C. koreensis*), were established on food contact surface as described previously in Section 2.4. Once the multispecies biofilm matured, TPU and SS coupons were transferred into Whirl-Pak bags along with 9 mL of BPW to achieve a 10-fold dilution and then sonicated for 1 min (Marouani-Gadri et al., 2009). The diluent was each plated onto MacConkey agar, Lactic Acid Bacteria Count Petrifilm (3 M™, Saint Paul, USA), TSA, and *Pseudomonas* agar + selective supplement (Oxoid-ThermoFisher, Nepean, ON). For biofilm mixture T1, the MacConkey plates were incubated at 37°C for 24 h for enumeration of *E. coli* O157:H7, and lactic acid bacteria Petrifilm were incubated at 30°C for 72 h for enumeration and to differentiate *C. piscicola* and *L. bulgaricus* (heterofermentative and homofermentative respectively). For the enumeration of strains co-cultured in multispecies biofilm T2, plates of TSA and MacConkey agar were used to determine the total bacterial count and *E. coli* O157:H7, respectively. Then, the total number of *C. koreensis* and *R. terrigena* can be calculated accordingly. For biofilm mixture T3, *Pseudomonas* agar was incubated at 37°C for 24 h for enumeration of *Pseudomonas*, while plates of MacConkey agar and TSA were used for determination of *E. coli* O157:H7 and *C. koreensis*, respectively. PCR was used to confirm bacterial colonies when the phenotype was not clear (Spilker et al., 2004; Torriani et al., 1999).

2.9 | Statistical analysis

All experiments were performed independently three times. Data were analyzed using the Statistical Analysis System Proc Mixed program (Cary, NC), with mean separation accomplished using the PDIFF option, and variance components were settled as covariance structure. For beef contaminated by O157:H7 biofilm from food contact surfaces, effects of contact surface, storage time, species, and humidity along with associated interactions were included in the model, with significance declared at $p \leq 0.05$. For multispecies biofilm biomass producing ability determination, a factorial model was applied to analyze the main effects of STEC species, pre-established multispecies biofilm strain combination, and their two-way interaction.

3 | RESULTS

3.1 | Multispecies biofilm formation on food contact surface and O157:H7 transfer to fresh beef

At a temperature of 25°C, the biofilm formed by the combination of *P. aeruginosa* and *C. koreensis* (T3) exhibited an O157:H7 transfer of 0.51 log CFU/cm^2. When compared to the control group with a transfer of 1.95 log CFU/cm^2, there was a significant reduction of

1.44 log10 ($p < 0.01$) in O157:H7 cells on beef (Figure 1a–c). On the other hand, the overall beef contamination caused by the biofilm mixture of *C. piscicola* + *L. bulgaricus* (T1) and *C. koreensis* + *R. terrigena* (T2) was not significantly different ($p > 0.05$) compared to the positive control (T4). Comparing the two surfaces, in general, O157:H7 beef contamination from TPU (1.76 $\log_{10}$ CFU/cm^2) was higher ($p < 0.01$) than from SS (1.17 $\log_{10}$ CFU/cm^2).

Overall, the beef contamination with O157:H7 decreased with biofilm aging, from 2.47 log (6 days) to 1.32 log (30 days) and 0.59 log (60 days) ($p < 0.01$). The 6-day *P. aeruginosa* + C. *koreensis* (T3) biofilms on TPU showed the highest antagonistic effect on O157:H7 transfer to beef regardless of humidity (2.47 $\log_{10}$ CFU/cm^2). Meanwhile, the highest beef contamination with O157:H7 was associated with 6 days *C. koreensis* + *R. terrigena* (T2) biofilms on TPU irrespective of humidity (4.10 $\log_{10}$ CFU/cm^2). After 60 days, no O157:H7 transfer to beef from *P. aeruginosa* + C. *koreensis* (T3) biofilms was detected, and beef contamination with O157:H7 from *C. koreensis* + *R. terrigena* (T2) on TPU surface (0.68 $\log_{10}$ CFU/cm^2) was lower ($p < 0.05$) than the positive control (T4) (1.49 $\log_{10}$ CFU/cm^2). In general, moist biofilms led to higher ($p < 0.01$) beef contamination (2.74 $\log_{10}$ CFU/cm^2) compared to dry biofilms (0.19 $\log_{10}$ CFU/cm^2).

At a temperature of 10°C, none of the tested multispecies biofilm combinations showed any significant effect on beef contamination with O157:H7 compared to the positive control (T4) (Figure 1d–f). The data illustrating biofilm formation, obtained through a crystal violet 96-well microplate assay conducted at both 25 and 10°C, has been presented in Figure S2. As for the surface type, the overall transfer of O157:H7 to beef from biofilms formed at 10°C was higher ($p < 0.01$) from TPU (1.00 $\log_{10}$ CFU/cm^2) than from SS (0.55 $\log_{10}$ CFU/cm^2). Furthermore, the overall contamination of beef with O157:H7 from moist biofilms (1.53 $\log_{10}$ CFU/cm^2) was higher ($p < 0.01$) than from dry biofilms (0.02 $\log_{10}$ CFU/cm^2). As the biofilms aged, transfer of O157:H7 to beef decreased ($p < 0.01$) from 1.31 $\log_{10}$ CFU/cm^2 on day 6 to 0.42 $\log_{10}$ CFU/cm^2 on day 60. Transfer of O157:H7 from 30 or 60 day dry biofilms to beef was not detected (data not shown). Beef samples exposed to 60 days old dry biofilms (10 & 25°C) that yielded negative enumeration results underwent *E. coli* O157:H7 detection, both prior to and following a 24-day enrichment period. The outcomes are shown in Table S1 for reference.

3.2 | *E. coli* O157:H7 survival rate within dry biofilms

In this study, the presence of O157:H7 was observed in measurable quantities after 6, 30, and 60 days in biofilms maintained under moist conditions at 10 and 25°C (Data not shown). However, in dry biofilms at both temperatures and at 30 and 60 days, O157:H7 was only detectable after enrichment (Table 2).At a temperature of 25°C, a synergistic effect was observed in the formation of biofilms on TPU ($p < 0.01$), which denotes a substantial disparity O157 recovery rate, particularly with the combinations of *C. koreensis* + *R. terrigena* (T2) and *C. piscicola* + *L. bulgaricus* (T1). T1 and T2 combinations

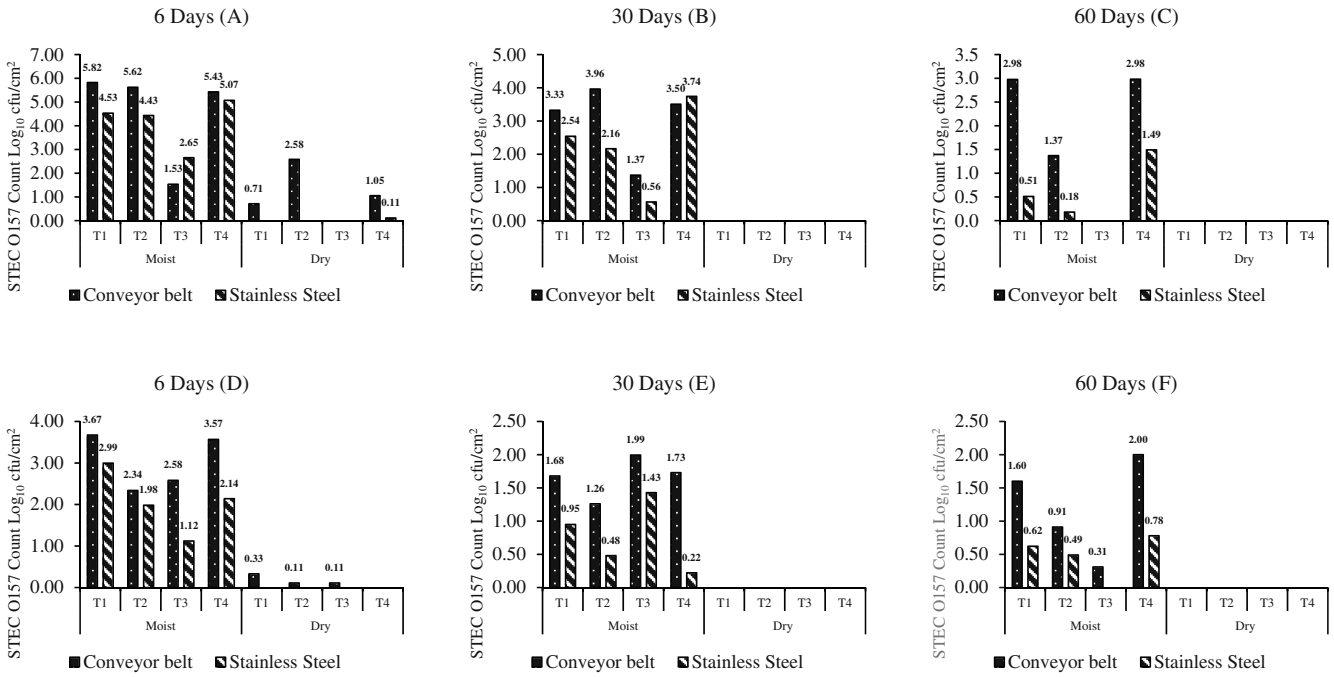

FIGURE 1 (a–c) Number of O157:H7 cells transferred to beef from moist or dry multispecies biofilms formed at 25°C for 6, 30, and 60 days. (d–f) Number of O157:H7 cells transferred to beef from moist or dry multispecies biofilms formed at 10°C for 6, 30, and 60 days. The four-strain combinations were (T1) *Carnobacterium piscicola* + *Lactobacillus bulgaricus*; (T2) *Comamonas koreensis* + *Raoultella terrigena*; (T3) *Pseudomonas aeruginosa* + *Comamonas koreensis*; and (T4) STEC O157:H7 positive control.

TABLE 2 Recovery of *E. coli* O157:H7 from dry multispecies biofilms after storage for 60 days with and without 24 h of enrichment.

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

Note: Biofilm was developed on either thermoplastic polyurethane (TPU) or 304 stainless-steel (SS) surfaces. The four-strain combinations were (T1) *Carnobacterium piscicola* + *Lactobacillus bulgaricus*; (T2) *Comamonas koreensis* + *Raoultella terrigena*; (T3) *Pseudomonas aeruginosa* + *Comamonas koreensis*; and (T4) STEC O157:H7 Control.
Abbreviations: ND, No *E. coli* O157:H7 recovery was detected (0/9).

significantly increased the survival rate of O157:H7 from 0/9 (T4) to 2/9 (T1) and 6/9 (T2) after enrichment, respectively (Table 2). Notably, when the same biofilm combinations were formed on SS surfaces at 25°C, they exhibited different survival rates after enrichment. T2 had a survival rate of 1/9 on SS, while T1 did not survive on SS (0/9), indicating that surface type had some impact on the survival and transfer O157:H7 (Table 2). In contrast, at 25°C the biofilm mixture *P. aeruginosa* + *C. koreensis* (T3) did not affect the survival of O157:H7 on either TPU (0/9) or SS (0/9), (Table 2).

At 10°C, the recovery rate of O157:H7 after enrichment on TPU was 8/9 for both T2 and T3 dry biofilms ($p < 0.01$), suggesting that its integration into mixed species biofilms enhanced O157:H7 survival (Table 2). Temperature also influenced O157:H7 recovery in T3 and T2 mixed biofilms, as recovery at 10°C (8/9 and 8/9) was higher than at 25°C (0/9 and 6/9 respectively). Interestingly no O157:H7 was recovered from *C. piscicola* + *L. bulgaricus* (T1) biofilms (0/9) at 10°C after enrichment. (Table 2). Similar to 25°C, at 10°C survival rate of O157:H7 on SS surface was reduced, where *C. koreensis* + *R. terrigena* (T2) had a 2/9 O157:H7 survival rate on SS, while no O157:H7 (0/9) was recovered from the *Lactobacillus* combination T1, the spoilage combination T3 (*P. aeruginosa* + C. *koreensis*), or T4 (O157:H7 monoculture) biofilms on SS (Table 2).

3.3 | 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 2) showed different cell density compared with their counterparts at 10°C (Figure 3). At 25°C, T2 and T4 biofilms displayed sporadic single cell layers on the TPU and SS, with a few aggregates of rod-shaped bacteria. At 10°C, *C. koreensis* + *R. terrigena* (T2) biofilms stored for 6 days showed a three-dimensional structure with redundant EPM matrix on the TPU and SS. In contrast, O157:H7 single-species biofilm (T4) was not observed on TPU at 10°C, and scattered clusters of bacteria in a monolayer were found on SS at this temperature.

At 25°C, although a relatively larger cell aggregate and more EPM production was observed in wet in T2 and T4 biofilms when compared with dry biofilms, they still formed monolayer biofilms (Figure 2). In the case of T1: *Carnobacterium piscicola* + *Lactobacillus bulgaricus* dry biofilms at 25°C showed a multilayer biofilm on TPU and a monolayer biofilm on SS surfaces (Figure S1). In contrast, at 10°C the dry multilayer biofilm of *C. koreensis* + *R. terrigena* (T2) completely covered the surface of TPU and SS, while wet multilayer T2 biofilms only partially covered the surface of TPU and SS

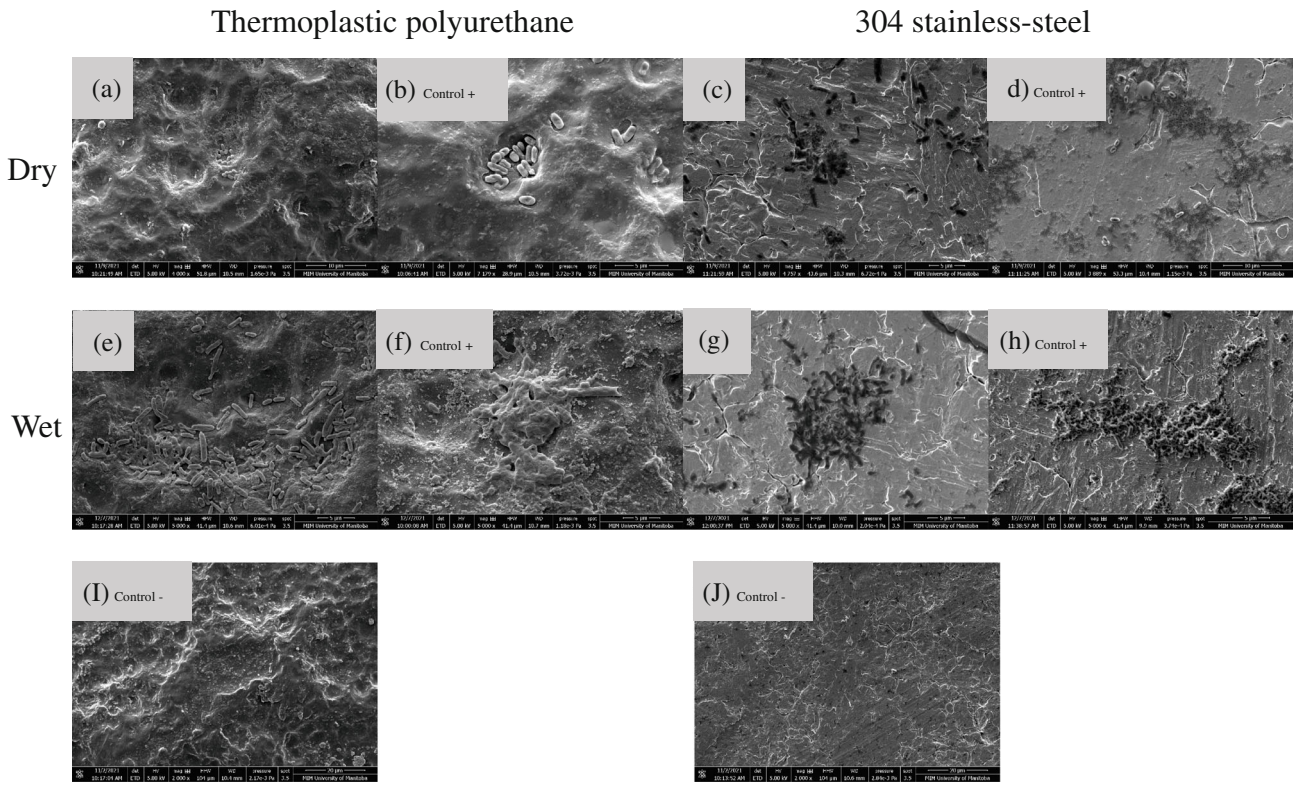

FIGURE 2 Scanning electron micrographs (SEM) of T2: *Comamonas koreensis* + *Raoultella terrigena* (a, c, e, and g) and T4: O157:H7 positive control (b, d, f, and h) dry and wet biofilms stored at 25°C for 6 days on the surface of thermoplastic polyurethane (TPU) and 304 stainless-steel (SS). SEM of (i) TPU and (j) SS coupons used as control negative, note that no bacteria are observed. Irregular spherical sag structure is observed on the TPU surface, accompany with isolated particulate matter.

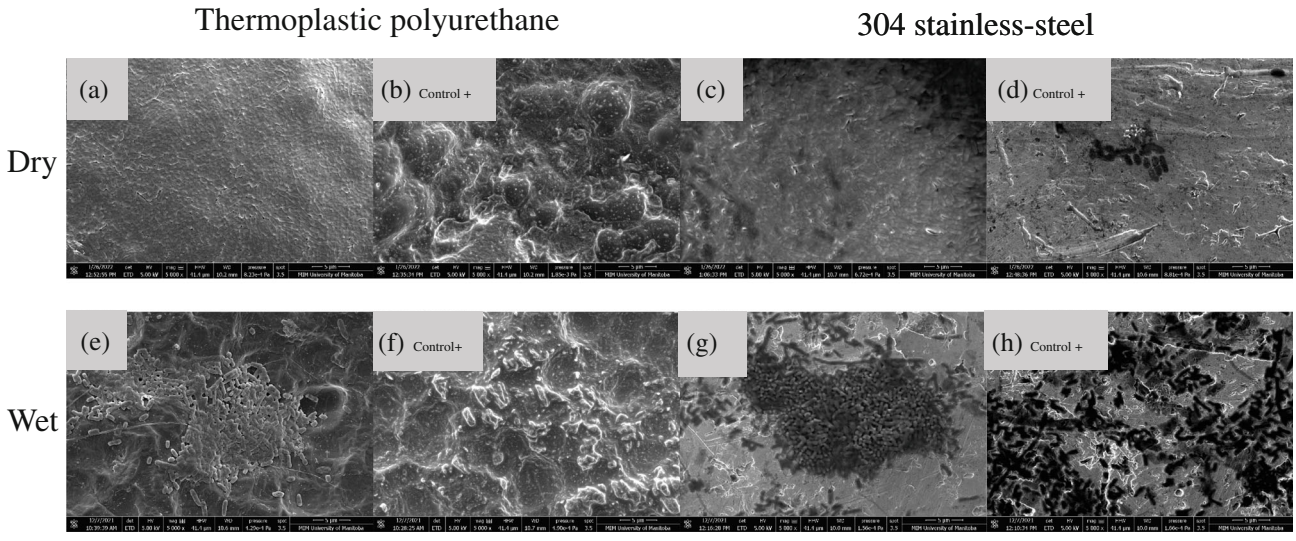

FIGURE 3 Scanning electron micrographs (SEM) of T2: *Comamonas koreensis* + *Raoultella terrigena* (a, c, e, and g) and T4: O157:H7 positive control (b, d, f, and h) dry and wet biofilms stored at 10°C for 6 days on a thermoplastic polyurethane (TPU) and 304 stainless-steel surface (SS).

(Figure 3). Nonetheless, when comparing moist and dry biofilms at 10°C, it was observed that O157:H7 single-species biofilm (T4) exhibited a greater cellular aggregation and increased extracellular polymeric matrix (EPM) production. On TPU surfaces, T2 (*C. koreensis* + *R. terrigena*) dry biofilms stored at 10°C for 60 days, showed a multilayer structure (Figure 4). Meanwhile, *P. aeruginosa* + *C. koreensis* (T3) dry biofilms displayed as individual bacterial cells embedded within an EPM that attached to the TPU surface.

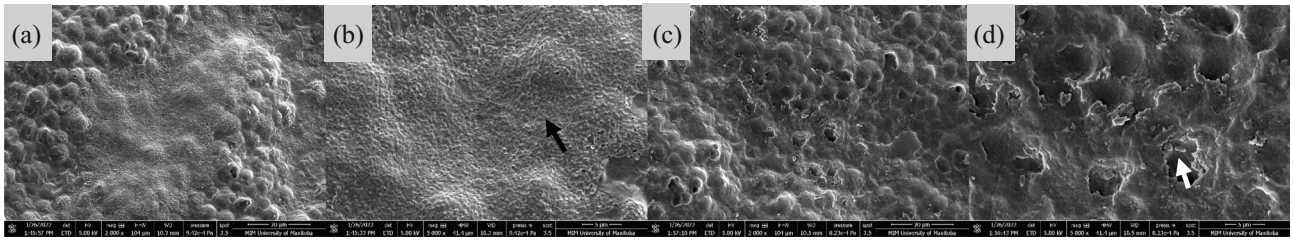

FIGURE 4 (a, b) *Comamonas koreensis* + *Raoultella terrigena* (T2), yellow arrows denote a rod-shaped cell covered with extracellular polymeric matrix; and (c, d) *Pseudomonas aeruginosa* + *Comamonas koreensis* (T3) dry biofilms stored at 10°C for 60 days on thermoplastic polyurethane (TPU). In figure (b) the rod-shape bacteria cell covered within extracellular polymeric matrix (EPM) matrix (black arrows) was observed. In figure (d) the individual bacterial cells adhering on coupons surface and coated with EPM (white arrows).

3.4 | Multispecies biofilm biomass production and bacteria enumeration

As shown in Figure 5a, at 25°C only the multispecies biofilm combination *C. piscicola* + *L. bulgaricus* (T1) exhibited stronger ($p < 0.01$) biomass production ability than O157:H7 alone (T4). On the other hand, there was no difference ($p > 0.05$) between *C. koreensis* + *R. terrigena* (T2), *P. aeruginosa* + *C. koreensis* (T3), and T4 observed for O157:H7.

At 10°C (Figure 5b), T1, T3, and T4 biomass production was lower ($p < 0.01$) than at 25°C, while T2 biomass production increased ($p < 0.01$). Besides, the biomass production ability of T2 at 10°C was higher ($p < 0.01$) than T1, T3, and T4.

As indicated in Table 3, the multispecies biofilm combination *P. aeruginosa* + *C. koreensis* (T3) showed *P. aeruginosa* dominated the biofilm-forming process at 25°C. In contrast, the biofilm mixture T3 at 10°C, *C. piscicola* + *L. bulgaricus* (T1) and *C. koreensis* + *R. terrigena* (T2) at either 10 or 25°C all exhibited more than one species participating in biofilm formation (Table 3). Results collected from TUP and SS surfaces indicated that the O157:H7 integrated into the pre-established biofilm and formed a true multi-species biofilm.

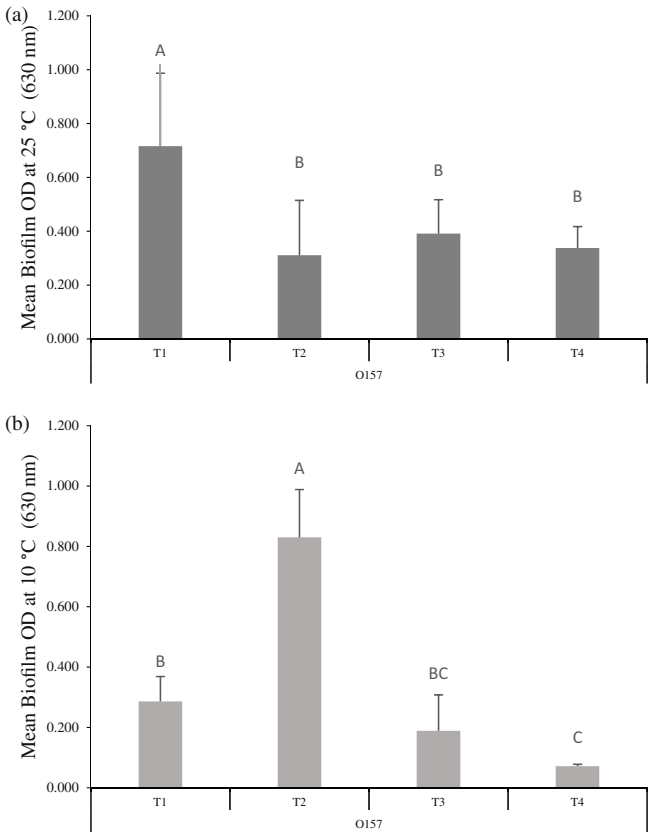

FIGURE 5 STEC O157:H7 containing multispecies biofilm biomass formation on microplates at (a) 25°C and (b) 10°C. Biomass formed by strain combination were determined in three replicate experiments. The biomass production differed ($p = 0.01$) with different biofilm strain combination among various temperature. Means with the same temperature with different capital letters differ ($p < 0.05$).

4 | DISCUSSION

4.1 | Multispecies biofilm interaction with O157:H7

Previous research has demonstrated that foodborne pathogens, such as *E. coli* O157:H7, have the capability to integrate into pre-existing multispecies biofilms found on surfaces that come into contact with food (Marouani-Gadri et al., 2009; Sofos & Geornaras, 2010). Nonetheless, the current body of knowledge lacks comprehensive insights into the influence of microbial flora, including spoilage bacteria, on the formation of O157:H7 biofilms and their potential to be transferred onto beef products. In the present study, *P. aeruginosa* + *C. koreensis* biofilm (T3) showed an antagonistic effect against O157:H7 at 25°C, reducing the transfer of O157:H7 cells to beef from 1.95 to 0.51 $\log_{10}$ CFU/cm^2 ($p < 0.001$) (Figure 1) these findings are consistent with the outcomes observed in mixed biofilms as illustrate in Table 3, where the presence of *Pseudomona* and *Comamonas* at 25°C reduced *E. coli* numbers below detectable levels. Similar results were reported for dual-species biofilms formed by STEC O157:H7 and *Salmonella typhimurium*, where it was found that bacterial species inoculated onto the contact surface first, were the dominant members within mature dual-species biofilms (Wang et al., 2013). Similar results were observed when *E. coli* O103:H2 (99-2076) was tested against the same multispecies biofilm tested here (Nan et al., 2022). It was found

TABLE 3 Biofilm formation by lactic acid bacteria and spoilage bacteria in monoculture or co-culture with *E. coli* O157:H7.

			Bacteria count (log_{10} CFU/cm^2)			
			25°C		10°C	
Surface	Strain combination	Strain	Mono-culture	Co-culture	Mono-culture	Co-culture
TPU	*C. piscicola* + *L. bulgaricus* (T1)	*C. piscicola*	6.5	4.0	8.0	5.5
		L. bulgaricus	4.9	3.5	3.1	2.0
		E. coli O157:H7	5.1	4.5	4.3	1.3
	C. koreensis + *R. terrigena* (T2)	*C. koreensis*	6.8	6.6	8.0	7.9
		R. terrigena	6.7	6.6	8.2	7.9
		E. coli O157:H7	5.1	6.4	4.3	0.0
	P.aeruginosa + *C. koreensis* (T3)	*P.aeruginosa*	6.4	6.8	8.0	6.0
		C. koreensis	6.8	0.0	8.0	7.9
		E. coli O157:H7	5.1	0.0	4.3	0.0
SS	*C. piscicola* + *L. bulgaricus* (T1)	*C. piscicola*	6.4	3.6	7.5	4.8
		L. bulgaricus	1.9	0.0	0.6	0.6
		E. coli O157:H7	3.3	4.6	0.0	1.5
	C. koreensis + *R. terrigena* (T2)	*C. koreensis*	5.9	6.1	7.0	7.7
		R. terrigena	6.4	6.1	7.7	7.7
		E. coli O157:H7	3.3	6.0	0.0	0.0
	P. aeruginosa + *C. koreensis* (T3)	*P.aeruginosa*	5.7	6.9	5.8	6.3
		C. koreensis	5.9	0.0	7.0	7.7
		E. coli O157:H7	3.3	0.0	0.0	0.0

Note: Standard deviations varied from 0.093 to 0.302. Biofilm was developed on either thermoplastic polyurethane (TPU) or 304 stainless-steel (SS) surfaces. The four-strain combinations were (T1) *Carnobacterium piscicola* + *Lactobacillus bulgaricus*; (T2) *Comamonas koreensis* + *Raoultella terrigena*; (T3) *Pseudomonas aeruginosa* + *Comamonas koreensis*; and (T4) STEC O157:H7 Control.

that *P. aeruginosa* + *C. koreensis* (T3) reduced O103:H2 transfer to beef by 2.54 log_{10} CFU/g at 25°C. The ability of *P. aeruginosa* + *C. koreensis* (T3) pre-established biofilms to inhibit the integration of O157:H7 and O103:H2 into multispecies biofilms is likely attributable to competition for essential nutrients, adhesion sites and temperature, which favors *Pseudomonas* ability to produce antimicrobial substances (Pang & Yuk, 2018; Wang, 2019). *P. aeruginosa* can produce harmful substances such as the pigment pyocyanin and pyoverdine, which likely impedes the ability of *E. coli* to thrive within multispecies biofilms as reported elsewhere (Das & Das, 2015; Pang et al., 2017). In this study, genomics analysis of *Pseudomona areruginosa* ATCC7700 showed that it harbors pyoverdine siderophores genes (PVDs). Pyoverdine production was observed during biofilm formation as a green pigment and intense fluorescence was observed when expose to UV light (data not shown), pyoverdine production was only observed at 25°C, which could partially explain O157:H7 reduction. Interestingly, at 25°C *P. aeruginosa* was the dominant species in the biofilm (Table 3). In contrast, at 10°C on SS & TPU, *C. koreensis* was the dominant species within the biofilm (Table 3) which have a lesser impact on O157 reduction at 10°C (Figure 1).

Pyoverdine is responsible for obtaining extracellular iron, and iron has been reported to be essential for biofilm formation within species of *P. aeruginosa* (Banin et al., 2005). In our research, pyoverdine production seems to be one of the factors that conferred *P. aeruginosa* with a competitive advantage. Interestingly, the inhibitory impact of *P. aeruginosa* + *C. koreensis* (T3) biofilms on O157:H7 wasn't observable at 10°C as illustrated in Figure 1. This discrepancy could be attributed to the relatively weakened biofilm-forming tendencies of *P. aeruginosa* and *C. koreensis* at the lower temperature, as indicated in Table 1 and in our previous study (Nan et al., 2022). In the case of *Pseudomona*, it was observed that pyoverdine was not produced at 10°C, showing that temperature is influencing the ability of *Pseudomona* to produce antimicrobial agents. It is also plausible that temperature could be influencing additional genes related to biofilm formation, virulence factors, and potentially other genes associated with bacterial resilience. Further investigation is warranted to comprehensively understand the broader impact.

In contrast to the results observed in the biofilms formed by *Pseudomonas* and *Comamonas*, the contamination of beef by O157:H7 originating from *C. piscicola* + *L. bulgaricus* (T1) and *C. koreensis* + *R. terrigena* (T2) biofilms formed at 25°C demonstrated a similarity ($p > 0.05$) to the transfer observed from the control, which is the O157:H7 single-species biofilm (T4). This suggests that these specific combinations did not lead to a reduction in O157:H7's ability to contaminate beef. At a temperature of 10°C, *C. korensis*, *R. terrigena*, and *C. piscicola* showed enhanced growth, leading to their dominance within the biofilms (Table 3). This dominance was corroborated by their growth curves in the planktonic state (unpublished data).

However, despite their prevalence in the biofilms, these bacterial communities did not demonstrate a significant reduction in the numbers of *E. coli* O157:H7, on the contrary it seemed to be beneficial for O157 survival, mainly in the dry state. It holds significance to recognize that zoonotic pathogens like *E. coli* O157 are unable to produce biofilms at 10°C, however, these pathogens could potentially become part of biofilms formed by other spoilage microorganisms as shown in this research. Nevertheless, the strength of these spoilage-associated biofilms might also be compromised by low temperatures, and weak biofilms should be easier to eliminate, which should be further investigated. These results suggest that the nature of interactions with O157:H7 are dependent on the bacterial species participating with mature biofilms (Wang, 2019). Prior research has examined the capacity of lactic acid bacteria (LAB) biofilms to proliferate within drainage systems in poultry processing facilities. These studies have shown that these biofilms are effective in managing *Listeria* contamination originating from floor drains (Pérez Ibarreche et al., 2014; Tong et al., 2013; Zhao et al., 2004). Furthermore, Chitlapilly Dass et al. (2020) emphasized the potential of LAB bacteria participating in multispecies biofilm formation to disrupt O157:H7's tolerance to sanitizers through antimicrobial agent production. This indicates the potential use of LAB bacteria for competitive exclusion, aiming to manage pathogenic bacteria within the meat industry. However, our present study yielded different results. We did not observe any antagonistic interaction between *E. coli* O157:H7 and the tested LAB strains in multispecies biofilms regarding beef contamination, as shown in Figure 1. Similar outcomes were observed with *E. coli* O103:H2 when combined with T1 (*C. piscicola* + *L. bulgaricus*), where the biofilm mixture did not affect the transfer of *E. coli* O103:H2 to beef (Nan et al., 2022). Interestingly, in this current research the recovery rate of O157:H7 from *C. piscicola* + *L. bulgaricus* (T1) multispecies biofilms at 25°C exceeded that of control positive samples, as outlined in Table 2. Conversely, in the first part of this study, we found that the recovery rate of O103:H2 from the T1 biofilm mixture was lower than that of the control positive samples, indicating some impact of LAB bacteria on STEC (Nan et al., 2022). These results suggest that the effect of LAB on STEC might be specific to certain strains.

These results emphasized the significant role of biofilms as potential reservoirs for pathogenic bacteria, presenting a formidable challenge for food safety. Furthermore, our findings reveal that the specific combinations of bacterial species found in food processing environments can influence the transfer of O157 to food items. Understanding these complex interactions is crucial for developing effective strategies to control and prevent the integration of pathogens into biofilms.

For instance, manual cleaning and sanitation procedures heavily rely on physical effort and mechanical disruptions to remove bacteria and organic nutrient layers that facilitate bacterial attachment and biofilm formation. However, situations of insufficient mechanical removal can arise due to various factors, and when coupled with damage to surfaces, they can escalate the probability of biofilm formation. Therefore, ensuring thorough mechanical removal of biofilms is vital for mitigating these risks.

4.2 | Beef contamination by O157:H7 varies between food contact surfaces

In the present study, the overall contamination of beef with O157:H7 from TPU was significantly higher than from SS, regardless of temperature (Figure 1). Midelet et al. (2002) studied the extent to which beef was contaminated by biofilms adhering to different food contact surfaces. *Listeria monocytogenes*, *Staphylococcus sciuri*, *Pseudomonas putida*, and *Comamonas* sp., all posed a greater risk of beef contamination when they were associated with TPU as compared to SS (Midelet & Carpentier, 2002). The higher contamination from biofilms on TPU has been attributed to its higher hydrophobicity as compared to SS, while previous studies demonstrated bacteria such as *Salmonella* and *Listeria* more readily formed biofilms on higher hydrophobic surfaces (Donlan, 2002; Sinde & Carballo, 2000). Consequently, biofilms are more likely to form on TPU than SS, and once formed, bacteria sloughing from biofilms on TPU may have a higher chance of contaminating beef (Figure 1). In our study, the recovery of O157:H7 from 60 days dry biofilms on TPU was higher than SS (Table 2), similarly, a previous study, a higher O103:H2 recovery was also observed from TPU than from SS (Nan et al., 2022). Therefore, the nature of the food contact surface could substantially impact the risk of beef contamination based on its propensity to support biofilm formation.

Considering that the influence of surface type is essential when assessing the risk associated with cleaning and sanitation practices, adapting cleaning protocols to account for surface type risk is essential, recognizing that TUP surfaces need more attention regarding bacterial attachment and biofilm formation can enhance the efficiency of cleaning and sanitation.

4.3 | Contamination of beef by O157:H7 differs with humidity and biofilm age

Studies elsewhere indicated that one of the difficult challenges for proper sanitation of beef fabrication equipment is removal of meat debris (Gill, 2009; Yang et al., 2017). This cleaning challenge can be attributed to the intricate topography of equipment surfaces, which tend to harbor meat particles within recesses and fissures, thus impeding their complete removal during cleaning procedures. Furthermore, the design of equipment and its conditions could exacerbate the cleaning predicament. Certain components of conveyor belts, such as hinges of jointed conveyor belts and intricate drive mechanisms, may necessitate equipment disassembly for thorough cleaning, adding to the complexity. Meat debris on the surface of equipment can protect bacteria from sanitizers and provide nutrients that promote biofilm formation (Gill, 2009; Yang et al., 2017).

After cleaning and sanitation, an effective drying of food contact surfaces is important to reduce microorganisms' proliferation. If biofilms are present on food contact surfaces they might dehydrate, however, bacteria within the matrix could be still viable, in addition time might also play a role on cell viability. Multiple studies have shown that desiccation can reduce the transfer of *E. coli* from meat

debris on beef fabrication equipment to beef (Gill & Landers, 2004; Youssef et al., 2013). In our study, no beef contamination was observed after exposure to 30 days old dry biofilms at both 10 and 25°C (Figure 1). Previous work by our group also found the desiccation reduced the contamination of beef from O103:H2 biofilms, a strain that can form strong biofilms (Nan et al., 2022). In contrast, O157:H7 from moist biofilms continued to serve as a source of beef contamination after 60 days storage at both temperatures (Figure 1). The standard commercial sanitation protocol employed by beef fabrication plants typically includes washing the equipment with hot water (40–50°C) for a few hours to remove residues, with the entire sanitation process taking up to 9 h (Wang et al., 2018). Although sustained temperatures of 10 or 25°C for extended periods are unlikely to occur throughout the cold chain, the routine use of hot water during sanitation in beef processing facilities creates a high humidity environment (Møretrø et al., 2010), which may contribute to the rehydration of bacterial biofilms. Additionally, *E. coli* harbored in meat debris or within fabrication equipment could survive the sanitation process and form biofilms at the warmer temperatures achieved during sanitation with hot water (Gill, 2009; Visvalingam et al., 2016). Importantly, any missed spots during the cleaning process can lead to the persistence of conditioning layers on equipment or environmental surfaces. These residual organic layers can serve as facilitators for bacterial colonization, exacerbating the risk of biofilm formation. It is crucial to recognize that biofilms not only impact the safety of the product, but can also affect its shelf life, particularly if composed of spoilage bacteria. Hence, consistent and proficient sanitation practices must achieve the thorough elimination of beef debris from both food-contact and non-food-contact surfaces within the processing equipment including conveyor belts and stainless-steel surfaces, along with thorough drying, is essential to minimize the risk of bacterial contamination during the beef fabrication process (Gill & Landers, 2004; Youssef et al., 2013).

4.4 | *E. coli* O157:H7 persistence in dry multispecies biofilm during long-term storage

Long-term storage periods can induce osmotic stress in bacteria within biofilms, potentially compromising bacteria viability (Iibuchi et al., 2010; Kim et al., 2008). In the present study, an increase in storage time was observed to reduce the transfer of O157:H7 to beef.

The primary role of the extracellular polymeric matrix (EPM) is to establish and maintain the structural integrity of biofilms, thereby shielding bacteria from a range of environmental stressors, including desiccation and antimicrobial agents (Barnhart & Chapman, 2006). Notably, EPM has been shown to serve as a robust protective barrier, safeguarding bacteria like *Pseudomonas* sp. and *Enterobacter* within biofilms from desiccation. EPS also plays an instrumental role in concentrating essential nutrients (Kim et al., 2008; Roberson & Firestone, 1992). Puga et al. (2016) reported that the nature of the EPM produced by individual species influences multispecies biofilm development and their functional properties (Puga et al., 2016). For instance, in *E. coli* O157:H7 and *Salmonella typhimurium* dual-species biofilms exhibited enhanced resistance to sanitizers as compared to single species biofilms. The authors proposed that this response was because more EPM in dual-species biofilms was produced (Wang et al., 2013). In this study, the multispecies dry biofilm T1 (*C. piscicola* + *L. bulgaricus*) and T2 (*C. koreensis* + *R. terrigena*) at 25°C, and T2 and T3 (*P. aeruginosa* + *C. koreensis*) at 10°C were observed to be surrounded by EPM structure (Figures 2–4). Furthermore, at 25°C the crystal violet staining technique confirmed a significantly higher ($p < 0.01$) biomass production capability for T1 biofilm mixture when compared to T4 at the same temperature, and higher ($p < 0.01$) biomass produced by T2 at 10°C than T4 (Figure 5). Likely extra EPM produced by *C. piscicola* + *L. bulgaricus* (T1) at 25°C and *C. koreensis* + *R. terrigena* (T2) at 10°C could facilitate the persistence of O157:H7 in desiccated multispecies biofilms. However, the biomass assay also revealed a similar ($p > 0.05$) biomass production by T2 (25°C) and T3 (10°C) compared with T4, respectively (Figure 5). Therefore, the increased persistence of O157:H7 in T2 (25°C) and T3 (10°C) multispecies biofilms cannot be solely attributed to biomass (EPM) production, necessitating further investigation into potential factors affecting O157:H7 persistence in dry multispecies biofilms, spatial distribution of O157:H7 on the composed strain and the chemical composition of the EPM (Chitlapilly Dass et al., 2020; Nan, 2022).

Upon contact with water or nutrients from beef products, dormant cells within these dry biofilms may reanimate and contaminate beef. This phenomenon was observed when O157:H7 was recovered from desiccated biofilms post-enrichment (Table 2). Interestingly, no O157:H7 was recovered from dry single-species biofilms (T4) after 60 days. Our previous study recovered O103:H2 (99-2076) from single-species biofilms at 25 and 10°C (Nan et al., 2022). Interestingly, O103:H2 (99-2076) did test positive for curli and cellulose-producing ability when tested in Congo red and LB-calcofluor agar, and it was also categorized as a strong biofilm former at 25°C, while the O157:H7 (1934) in the current study did not express curli—cellulose production. It was an intermedia biofilm former at 25°C. Hence, the curli and cellulose-producing ability might contribute to O103:H2 single-species biofilms having a more EPM production, contributing to biofilm thickness and likely enhancing its resistance to desiccation (Adator et al., 2018; Gualdi et al., 2008; Kumar & Anand, 1998).

Results also showed that O157:H7 recovery varies with temperature. More O157:H7 was recovered from *C. koreensis* + *R. terrigena* (T2) and *P. aeruginosa* + *C. koreensis* (T3) multispecies biofilms at 10°C then at 25°C (Table 2). In contrast, recovery of O157:H7 from *C. piscicola* + *L. bulgaricus* (T1) multispecies biofilms was higher at 25°C than 10°C. This suggests that the ability of O157:H7 to persist in biofilms at various temperatures differs depending on the bacterial species and cohabitants within biofilms. Within beef processing facilities, low environmental temperatures (5–15°C) are strategically employed to restrict the proliferation of enteric pathogens and spoilage bacteria on both contact surfaces and within food products (Ma et al., 2019, 2020). Nonetheless, it's important to note that not all bacterial species exhibit equivalent sensitivity to temperature fluctuations or changes in humidity. In the current study context, when *E. coli* O157:H7 (strain 1934) was subjected to microplate testing, it was

classified as a weak-biofilm former at 10°C (Table 1). However, subsequent trials on thermoplastic polyurethane and stainless steel revealed that this strain demonstrated the ability to transfer cells from biofilms to beef and successfully integrate and persist within multispecies biofilms (Figures 1 and 3). Within the food industry, strong biofilm-forming pathogenic bacteria are generally perceived as posing a more significant food safety risk compared to weak biofilm formers, primarily due to their increased likelihood of acting as contaminants during meat processing (Keskinen et al., 2008). Interestingly, the introduction of the weak biofilm forming O157:H7 to pre-formed biofilms of *C. koreensis* + *R. terrigena* (T2) on TPU and SS surfaces at 10°C resulted in an enhanced persistence of O157:H7 during storage at 10°C (Table 2). Consequently, it is inferred that spoilage bacteria could potentially impact food safety by modulating the survival and persistence of foodborne pathogens within multispecies biofilms.

Given the diversity of foodborne pathogens of concern in the food industry and lactic acid bacteria, selecting a biocontrol strategy, such as introducing LAB bacteria, necessitates assessing the effectiveness of these measures against all targeted bacterial species. Each facility might require tailored interventions, considering the varied interactions and strains. Because of the complexity of our experimental setup, we only looked at how two LAB strains and three spoilage strains behave. This is a limitation of our study, considering that many different microorganisms exist in food processing environments. However, our results clearly show that these groups of microorganisms play an essential role in how harmful pathogens such as STEC continue to exist, and it deserves more research.

The current data presented in this manuscript complements our previous work; in this study, we focused on O157:H7 strain 1934, an intermediate biofilm former. *E. coli* O157:H7 1934 strain was subjected to whole genome sequence (data no shown) and it was found that it carries the genes previously reported to be linked to curli (*csg*A, *csg*B, *csg*C *csg*D, *csg*E, *csg*F, *csg*G) and cellulose (*bcs*E, *bcs*F, *bcs*G, *bcs*Q) production (Acheson et al., 2021; Hammar et al., 1995), yet intriguingly, it did not express these traits under the tested conditions, which need to be explored. In contrast, our previous research, conducted by Nan et al. (2022), examined a non-O157 O103 strain known for robust biofilm formation, characterized by the expression of curli and cellulose (Nan et al., 2022). By comparing these distinct strains within the same biofilm combinations, we observed variations in their survival behaviors under the experimental conditions tested in this research.

5 | CONCLUSIONS

The results obtained in this study demonstrate that SP and LAB bacteria commonly found in the beef processing environment can affect *E. coli* O157:H7 persistence and survival. The biofilm mixture *P. aeruginosa* + *C. koreensis* (T3) had antagonistic effects against O157:H7 at 25°C, reducing its transfer to beef. This effect was attributed to competition for nutrients, adhesion sites, temperature, and the production of harmful substances by *P. aeruginosa*. In contrast, other pre-existing biofilms did not affect the transfer of O157:H7 to beef, highlighting the species-specific nature of these interactions. Interestingly, dry biofilm mixtures of *C. piscicola* + *L.bulgaricus* (T1), *C. koreensis* + *R. terrigena* (T2), and *Pseudomona* + *Comamonas* (T3) enhanced O157:H7 survival over a range of temperatures after 60 days of storage, perhaps due to EPM production or other factors such as genetic makeup. The contamination of beef by O157:H7 varied depending on the food contact surface, with TPU surfaces showing higher contamination rates than stainless steel. Temperature and humidity also played significant roles, with higher humidity promoting bacterial transfer and subsequent contamination of beef. Long-term storage of dry multispecies biofilms reduced the transfer of O157:H7 to beef, but the recovery rate varied depending on the bacterial species within the biofilm. The production of extracellular polymeric matrix (EPM) by certain bacterial species within biofilms influenced the persistence of O157:H7. The intermediate biofilm forming O157:H7 strain also demonstrated enhanced endurance when introduced to pre-formed biofilms of spoilage bacteria. These findings highlight the importance of understanding the complex dynamics of biofilm interactions in order to develop effective strategies for controlling and preventing the integration of pathogens into biofilms in food processing environments. Thorough, effective cleaning, and sanitation, as well as consideration of surface type, temperature, and humidity factors, are crucial for minimizing the risk of bacterial contamination during food processing. Further research is needed to explore the factors influencing pathogen persistence in multispecies biofilms and the potential application of biocontrol strategies for pathogen management in the meat industry.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the manuscript/Supplementary Material, further inquiries can be directed to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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