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# Ability of Shiga toxigenic *Escherichia coli* to survive within dry-surface biofilms and transfer to fresh lettuce

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ABSTRACT

Biofilms are known to play important roles in bacterial survival and persistence in food-processing environments. This study aimed to determine the ability of the top 7 STEC serotypes to form biofilms on polystyrene (POL) and stainless steel (SS) plates and to quantify their survival and transfer from dry-surface biofilms to lettuce pieces. The ability of 14 STEC strains to form biofilms on these two materials at different exposure times and temperatures was assessed using crystal violet, Congo red and SEM. At 10 °C all serotypes were weak biofilm producers on both surfaces. In contrast, serotypes O45-040, O45-445, O103-102, O103-670 and O157-R508 were strong biofilm producers at 25 °C. Strains O103-102, O103-670, O111-CFS, O111–053 and O157:H7-R508 were expressers of curli. Under scanning electron microscopy, strains O103-670, O111-CFS, O157-R508, and O121-083 formed more discernible multilayer, mature biofilms on SS coupons. Regardless of the surface (POL/SS), all STEC strains were able to transfer viable cells onto fresh lettuce within a short contact time (2 min) to varying degrees (up to 6.35 log cfu/g). On POL, viable cell of almost all serotypes exhibited decreased detachment ($p = 0.001$) over 6 days; while after 30 days on SS, serotypes O45-040, O103-102, O103-670, O111-053, O111-CFS, O121-083, O145-231 O157:H7-R508 and O157:H7-122 were transferred to lettuce. After enrichment, all 14 STEC strains were recovered from dry-surface biofilms on POL and SS plates after 30 days. Results demonstrated that the top 7 STEC remained viable within dry-surface biofilms for at least 30 days, transferring to lettuce within 2 min of exposure and acting as a source of adulteration.

## 1. Introduction

Pathogen contamination of foods, related disease outbreaks, and food recalls are reoccurring problems in the food industry. The Codex committee on food hygiene has identified leafy vegetables as one of greatest microbiological hazards within food production (Food and Agriculture Organization of the United Nations, 2008) and numerous countries have identified pathogens on fresh produce as a public health risk that restricts trade in these goods (Food and Agriculture Organization of the United Nations, 2008). In the US between 1973 and 2012, 606 outbreaks, 20,003 illnesses, 1030 hospitalizations and 19 deaths were associated with the microbial contamination of leafy vegetables (Herman et al., 2015). Among the foodborne pathogens identified, Shiga-toxigenic *Escherichia* (STEC) was commonly associated with fresh produce, especially serotypes O157, O121, O26 and O145, which have been reported to be responsible for 18% of outbreaks linked to leafy greens (CDC, 2017; Gould et al., 2013).

Contamination of leafy greens is particularly concerning because they are usually consumed raw. In addition, it has been reported that in the case of fresh cut leafy greens, there is an increased risk of contamination due to the exposure of sites that are more ammendable to bacterial colonization. For example, it has been shown that *E. coli* O157:H7 prefers to attach to the cut edges of lettuce rather than to the intact leaf (Boyer et al., 2007; Herman et al., 2015; Matthews, 2006; Takeuchi et al., 2000). Contamination of leafy greens can occur at pre- and/or post-harvest stages of production. During processing the presence of foodborne pathogens in the environment may play an important role in adulteration. In the fresh produce industry, environmental sampling for foodborne pathogens is less comprehensive than in the meat industry, probably because the beef industry has a longer history of outbreaks linked to *E. coli* O157:H7. Intensive sampling carried out by the beef industry has revealed the occurrence of “high event periods” (HEP) involving STEC *E. coli* (Stanford et al., 2017). The U.S. Department of Agriculture Food Safety and Inspection Service

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(FSIS, 2014) described HEPs as "periods in which slaughter plants identify a high number of trim samples positive for STEC (or virulence markers) in production lots originating from a common source", but the causes and mechanism behind HEPs in processing plants remains largely unknown (Arthur et al., 2014; Wang et al., 2014). Recent studies have suggested that biofilms may be a chronic source of the STECs responsible for HEPs (Wang et al., 2014). Bacteria occurring within biofilms are generally more resistant to antimicrobial agents and tend to survive sanitation treatments better than when in planktonic form (Li et al., 2012; Wang et al., 2012). A very low infectious dose (< 100 organisms) of STECs can cause illness (Strachan et al., 2001; Teunis et al., 2004), as such, minute surface contamination of work areas can result in serious infections.

The role of biofilms in fresh fruit and vegetable recalls and outbreaks has been extensively reviewed by Annous et al. (2009). Generally, considerable similarity exists between meat and produce processing operations, as both receive perishable raw materials and store finished products. Food contact surfaces such as stainless steel, plastics, and glass are common to both meat and produce operations. Likewise, low temperature storage is used to inhibit microbial growth by both industries. Therefore, it seems likely that HEPs might be associated with biofilm formation in both processing environments and thereby it plays an important role in food contamination (Gibson et al., 1999; Liu et al., 2013; Vogeleer et al., 2014).

Research on the capacity of STEC to form biofilms and transfer from food contact surfaces and to resist sanitizers has been conducted by testing biofilms *in vitro* at solid/liquid interfaces (Ban and Kang, 2016; Poulsen, 1999; Wang et al., 2016a; Wang et al., 2016b). However, these conditions may not always represent the conditions encountered by foodborne pathogens in food processing plants. To our knowledge no research has studied the transfer of STEC from dry-surface biofilms formed on food contact materials to leafy greens or STEC survival within dry-surface biofilms. There is a need to understand STEC survival within organic matter on food contact surfaces, not only at liquid interfaces, but also under dry conditions with different storage temperatures. Such information will facilitate risk assessment of the contribution of STEC biofilms to fresh produce contamination. Therefore, the objectives of this study were: i) to evaluate the biofilm forming abilities of 7 STEC serotypes on polystyrene and stainless steel surfaces at different storage times and temperatures; ii) to evaluate STEC transfer from dry-surface biofilms to lettuce under these conditions and iii) to determine the ability of STEC to survive within the dry-surface biofilms.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

Fourteen STEC strains belonging to the top 7 STEC serotypes, originating from humans or cattle, were used in this study (Table 1). *E. coli* strains were cultured on MacConkey agar (Oxoid Ltd., Basingstoke, UK) overnight at 37 °C. One colony was transferred from each plate into 9 mL tryptic soy broth (TSB; EMD Millipore Corp., Billerica MA, US) and incubated overnight to a density of $10^8$ cfu/mL. The cultures were diluted by $10^{-2}$ cfu/mL in low salt Lennox broth (LB-LS; Fisher Scientific, Waltham MA, US) and subsequently used in the biofilm assays.

### 2.2. Biofilms forming ability of STEC strains

#### 2.2.1. Biofilm formation assessment using crystal violet

The *in vitro* biofilm forming ability of each strain on polystyrene plates (POL) was determined as described by Wang et al. (2012) with some modifications. Briefly, overnight cultures of *E. coli* grown in trypticase soy broth (TSB) were diluted in LB-LS to $10^6$ cfu/mL. Each well in a 96 well plate (Corning Inc., Corning, NY, US) was inoculated with 20 µL of diluted culture. Different groups of microplates were prepared for testing at different storage times and temperatures. A group of plates were statically incubated at 10 °C and 25 °C and examined at days 4 and 6. Subsequently, fluid associated with biofilms was gently aspirated, and biofilms were washed three times with phosphate buffered saline (PBS, pH 7.4) before being air dried for ~30 min. Each well was then stained with 100 µL 0.1% crystal violet for 20 min, after which the plates were re-washed twice with PBS and dried for an additional 30 min. Crystal violet was extracted from at least 10 replicate wells and quantified at an optical density of 630 nm ($OD_{630nm}$) using a microplate reader (ELx800, BioTek Instruments, Inc., Winooski, VT, USA). The experiment was repeated 3 times. Serotypes previously characterized for their biofilm forming capacity were used as controls, with 011175 being a weak biofilm former and 0182 being a strong biofilm former on a polystyrene surface *in vitro*. In addition, LB-LS without *E. coli* served as a negative control.

**Table 1**
List of *Escherichia coli* strains investigated for biofilm formation.

| Strain | ID | Origin |
|---|---|---|
| O26:H11 | EC19960464[a] | Bovine/feces |
| O26:H11 | 00-3941 | Human |
| O45:H2 | EC19940040[a] | Bovine/feces |
| O45:H2 | 04-2445 | Human |
| O103:H2 | 01-6102 | Human |
| O103:H2 | EC20010670[a] | Bovine/feces |
| O111:NM | CFS3 | Human |
| O111:NM | EC20030053[a] | Bovine/feces |
| O121:H19 | EC20040083[a] | Bovine/feces |
| O121:H19 | 03-2832 | Human |
| O145–4699 | 03-4699 | Human |
| O145:NM | EC20020231[a] | Human |
| O157:H7 | EO122 | Bovine/feces |
| O157:H7 | R508 | Bovine/feces |

[a] Strains obtained from Lethbridge Research and Development Centre, Agriculture and Agri-food, Canada; the rest of the STEC strains were obtained from the University of Manitoba culture collection. All strains in the present study are denoted by their serotype (first three values under '**Strain**') followed by the last three or more number/letters of their "ID" given above.

#### 2.2.2. Biofilm formation assessment by Curli expression

Curli expression was assessed as described by Boyer et al., 2007. Each strain (10 µL) was plated onto yeast extract casamino acids agar (10 g/L casamino acids, 1 g/L yeast extract, and 20 g/L agar) without salt and supplemented with 40 µg/mL Congo red dye (Sigma Aldrich, St. Louis, MO.) and 20 µg/mL Coomassie brilliant blue dye (Sigma Aldrich), (YESCA-CR). Agar plates were incubated at 28 °C for 48 h. Those colonies which appeared red were indicative of curli expression. This experiment was repeated 3 times/strain.

### 2.3. Biofilm formation on food contact surfaces and STEC transfer from dry-surface biofilms

#### 2.3.1. Polystyrene plates, coupon preparation, biofilm formation, and STEC transfer

Sterile 6-well culture (non-treated) POL plates (VWR®, VWR International, LLC) were used. An overnight culture of each strain was transferred into 18 mL of sterile LB-LS and diluted 100-fold to $10^6$ cfu/mL. A bacterial suspension (2.5 mL) of each serotype (Table 1) was transferred into each well of the polystyrene plate in duplicate. Negative control wells only contained LB-LS. Inoculated plates were assigned to treatment groups and statically incubated for 6 days at 10 °C and 25 °C to allow biofilm formation. After incubation, the supernatant from each well was removed by aspiration, and the wells were washed three times with 2.5 mL of sterile PBS (pH 7.4) to remove loosely attached cells. All plates were allowed to air dry at room temperature for 4 h, covered and then stored at room temperature until tested. Plates were assigned to different groups according to the storage period. STEC