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Curli fimbriae confer shiga toxin-producing *Escherichia coli* a competitive trait in mixed biofilms

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) is one of the most common causal agents of foodborne illness linked to fresh leafy vegetables. Here, we investigated the impact of spinach-associated microorganisms on proliferation and biofilm formation of STEC O157:H7 on stainless steel surfaces at temperatures related to produce production and postharvest processing environments. Although a proliferation of inoculated pathogen cells in spinach leaf wash water was detected at all temperatures examined, the impact of spinach-associated microorganisms on the proliferation of *E. coli* O157:H7 was observed at 10 °C and 26 °C, but not at 4 °C. The inhibition of *E. coli* O157:H7 growth by spinach-associated microorganisms indicated a competition between the pathogen and spinach indigenous microflora. A significant decrease of the pathogen population in mixed biofilms was observed only at 26 °C for curli-deficient strain MQC43, but not for curli-expressing strain MQC57. Deletion of curli genes in a curli-expressing strain resulted in a phenotype similar to that of MQC43 in mixed biofilms; however, this deficiency was rescued when curli biogenesis was restored in the curli-deletion mutant strain. Our data support that curli confer *E. coli* O157:H7 a competitive trait in mixed biofilms, presumably through the interaction between STEC and the biofilm-proficient microorganisms associated with spinach leaves.

1. Introduction

Leafy vegetables are commonly colonized by diverse microorganisms and can become contaminated with human pathogens at various stages of production, processing, storage, and distribution (Gil et al., 2015; Smith et al., 2018). In preharvest environments, contaminated water or tools, dust containing manures, wildlife animals, and insects can potentially serve as the sources of contamination (Alegbeleye et al., 2018; Brandl, 2006; Gutierrez-Rodriguez and Adhikari, 2018). At the postharvest level, contamination may result from water used for washing and processing produce, workers, or cross-contamination from other food (Gomez-Lopez et al., 2013; Smolinski et al., 2018). Additionally, inadequate storage temperature, minimally processed vegetables, and mechanically or biologically damaged leafy greens may facilitate amplification of pathogens on produce (Alegre et al., 2010; Brandl, 2008; Duvenage and Korsten, 2016; Huang et al., 2015; Koukkidis et al., 2017; Luo et al., 2010; Sim et al., 2013), and pose a high health risk to consumers.

Shiga toxin-producing *Escherichia coli* (STEC) is one of the main causal agents of foodborne illness outbreaks linked to fresh leafy vegetables. Formation of biofilm by STEC on fresh produce enhances survival of STEC since biofilm-associated cells are better at coping with environmental stresses including nutrient limitations (Carter and Brandl, 2015; Watnick and Kolter, 2000; Yaron and Romling, 2014). Similarly, formation of biofilm by STEC on abiotic surfaces enhances persistence of this pathogen in postharvest environments since biofilm-associated cells are more difficult to remove and more resistant to inactivation when compared to planktonic cells; thus they are generally more resistant to antimicrobial washes used in the produce industry (Costerton et al., 1987; De Beer et al., 1994; Ryu and Beuchat, 2005). Biofilms of STEC were reported on various produces and abiotic surfaces (Han et al., 2017; Nagy et al., 2015, 2016; Vogeleer et al., 2014; Wright et al., 2017; Ximenes et al., 2017), which may serve as a source of cross-contamination in postharvest and food-processing environments (Adator et al., 2018).

STEC strains produce an array of adhesive structures and proteins for colonization of their animal hosts, many of which promote colonization and biofilm formation by STEC on plants and abiotic surfaces (Carter et al., 2018; Lloyd et al., 2012; Matthysse et al., 2008; McWilliams and Torres, 2014; Saldana et al., 2011). Curli are the long aggregative fimbriae produced by many *Enterobacteriaceae* (Barnhart and Chapman, 2006). Curli mediate binding to, and invasion of epithelial cells in *E. coli* O157:H7 (Uhlich et al., 2002) and are also implicated in the attachment of pathogens to alfalfa sprouts (Barak et al.,

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2005; Torres et al., 2005) and leafy greens (Fink et al., 2012; Macarisin et al., 2012). Curli also mediate the direct contact between bacterial cells and abiotic surfaces (DeBenedictis et al., 2016). Recently, we reported a conditional role of curli in *E. coli* O157:H7. Curli fimbriae significantly enhanced the initial attachment of *E. coli* O157:H7 to spinach leaves and stainless steel and promoted *E. coli* O157:H7 biofilm formation on stainless steel in a culture condition dependent manner; however, curli fimbriae exhibited a tempering effect on adherence of *E. coli* O157:H7 to bovine recto-anal epithelial cells, implying a niche dependent role of curli in this pathogen (Carter et al., 2016; Kudva et al., 2017). To better understand the persistence of STEC in the produce production continuum, here we investigate the contribution of curli fimbriae during the establishment of mixed biofilms by *E. coli* O157:H7 and spinach leaf-associated microflora on stainless steel under the conditions related to produce field production and postharvest processing environments.

2. Material and methods

2.1. Bacterial strains, plasmids, and growth media

The *E. coli* strains and mutants used in this study and their sources are listed in Table 1. Strains were grown routinely in LB broth (Lennox) at 37 °C, or on LB-no salt (LBNS) agar at 26 °C, a condition known to induce curli biogenesis. Antibiotics were used at the following concentrations: kanamycin (Km), 25 μg/ml; chloramphenicol (Cm), 15 μg/ml; and rifampicin (Rif), 25 μg/ml for maintenance and 100 μg/ml for selection.

2.2. Cloning and complementation analysis

Primers used for amplification and sequencing are listed in Table S1. A DNA fragment containing the *csgBAC* operon was PCR amplified from genomic DNA of *E. coli* O157:H7 strain RM6535, digested with the restriction enzymes KpnI and HindIII and cloned into the plasmid pBBR1MCS, resulting in pXQ34 (Table 1). *E. coli* DH5α was used as a host strain for plasmids. The sequence-confirmed plasmid pXQ34 was transformed into the target strains by electroporation. The curli-deletion mutant strain was described previously (Carter et al., 2011). Briefly, the gene encoding curli subunit protein CsgA was replaced with a kanamycin cassette using the standard Lambda Red-mediated gene replacement method (Datsenko and Wanner, 2000).

2.3. Preparation of spinach wash water

The spinach wash water was prepared as described previously (Carter et al., 2016). Briefly, approximately 50 g of organic baby spinach purchased from a local retail store on the day of the experiment were placed in a Ziplock bag containing 250 ml KP buffer (10 mM K_2PO_4, pH 7.0). The leaves were sonicated in an ice-water bath for 10 min using a Fisher Scientific FS30 Ultrasonic Cleaner (Ultrasonic power, 130 W; operating frequency, 40 kHz) followed by incubation on a Belly Dancer® undulating orbital shaker at room temperature for 15 min. The leaf wash water was then filtered through a double layer of cheesecloth to remove plant debris. The filtrate, hereafter called spinach wash water (SWW), was used for pathogen inoculation. The sterile SWW was made by passing the SWW through a 0.22 μm filter (Millipore).

2.4. Mixed biofilm on stainless steel coupons (SSC)

Biofilm formation on stainless steel surfaces was carried out as described previously (Carter et al., 2012, 2016). Briefly, single colonies of each strain and mutant were grown on LBNS agar plates supplemented with 25 μg/mL rifampicin at 26 °C for 2–3 days to induce the curli biogenesis. The cells were removed from the plates using a cotton swab and resuspended in 5 mL KP buffer. The resulting cell suspension was used for inoculation.

Precut stainless steel coupons (1.0 × 0.5 inch) (SSC) were purchased from Speedy metals LLC and cleaned as described previously (Rivas et al., 2007). The clean coupons were finally rinsed once with Millipore water and air-dried prior to autoclaving. A sterile coupon was placed in a culture tube containing two ml of sterile SWW (for pure biofilm) or non-sterile SWW (for mixed biofilm) inoculated with 5×10^6 cells/ml of *E. coli* O157:H7 by adding two μl of overnight culture diluted in KP buffer at a concentration of OD_{600} at 1.0. The actual concentration of inoculum was determined by enumerating the colony forming units (CFUs) of 10-fold serial dilutions of the cell suspension on LB agar plates supplemented with 100 μg/ml rifampicin. A culture tube containing 2 ml of sterile SWW without pathogen inoculation was used as a control to monitor the sterility of filtered SWW. A culture tube containing 2 ml of non-sterile SWW without pathogen inoculation was used to monitor the spontaneous rifampicin-resistant mutants of natural spinach leaf-associated bacteria. The tubes were incubated statically at 4 °C, 10 °C, or 26 °C for 10 days. On a sampling day, the coupon was removed and rinsed twice with sterile KP buffer and placed in a new culture tube containing 2 ml KP buffer. The culture tubes were placed in an ice-water bath and sonicated for 10 min using a Fisher Scientific FS30 Ultrasonic Cleaner followed by vortex to release cells attached to coupon as described previously (Carter et al., 2016). The combination of sonication and vortex did not impact the recovery of either MQC43 or MQC57 based on plate counts. The population size of *E. coli* O157:H7 on SSC was determined by enumerating colonies on LB agar plates containing 100 μg/ml of Rifampicin and expressed as the

Table 1
Strains and plasmids used in this study.

Strains	Antibiotic resistance[a]	Characteristics	Source or Reference
E. coli O157:H7			
RM6067	$Km^SRif^SCm^S$	A bagged-spinach isolate linked to the 2006 US spinach-associated outbreak	Carter et al. (2011)
MQC10	$Km^SRif^SCm^S$	Curli-deficient variant of RM6067 (RM6067W)	Carter et al. (2011)
MQC43	$Km^SRif^RCm^S$	A spontaneous rifampicin resistant mutant of MQC10	This study
RM6535	$Km^SRif^SCm^S$	A lettuce isolate linked to a bagged iceberg lettuce-associated outbreak in 2006	Carter et al. (2011)
MQC17	$Km^SRif^SCm^S$	Curli-expressing variant of RM6535 (RM6535R)	Carter et al. (2011)
MQC57	$Km^SRif^RCm^S$	A spontaneous rifampicin resistant mutant of MQC17	Carter et al. (2016)
MQC114	$Km^RRif^SCm^S$	*csg*A deletion mutant of MQC17	Carter et al. (2011)
MQC946	$Km^RRif^RCm^S$	A spontaneous rifampicin resistant mutant of MQC114	Carter et al. (2016)
MQC1080	$Km^RRif^RGm^R$	MQ946 transformed with plasmid pXQ34	This study
MQC1084	$Km^RRif^RCm^R$	MQ946 transformed with plasmid pBBR1MCS	This study
Plasmids			
pBBR1MCS	Cm^R	Expression vector for complementation analysis	Kovach et al. (1995)
pXQ34	Cm^R	The *csgBAC* operon in *E. coli* O157:H7 strain RM6535 was cloned into vector pBBR1MCS	This study

[a] $Km^SRif^SCm^S$, sensitive to kanamycin, rifampicin, and chloramphenicol; Rif^R, rifampicin resistance; Km^R, kanamycin resistance; Cm^R, chloramphenicol resistance.

percent of initial inoculum. For control tubes containing sterile SWW, a 50 µl aliquot was retrieved at each sampling time and plated on LB agar plates; for control tubes containing non-sterile SWW, a 50 µl aliquot was retrieved at each sampling time and plated on LB agar plates supplemented with 100 µg/ml rifampicin, followed by incubating at 26 °C for three days and then checked for any visible colonies. Four biological replicates were included in each experiment and each experiment was repeated at least once independently.

2.5. Statistical analysis

Statistical analysis was computed with Prism 7.0 (GraphPad Software). Unpaired t-test was used to detect if the total population of STEC differed significantly from the initial inoculum or if the biofilm population changed significantly during the 10-day incubation. Multiple t tests followed by the Holm-Sidak method to correct for multiple comparisons were used for comparison between *E. coli* O157:H7 strains. Two-way ANOVA followed by Sidak's multiple comparisons test or Turkey's multiple comparisons test with α = 0.05 was used to assess if temperature, incubation time, and spinach leaf-associated microorganisms impact the proliferation and biofilm formation of *E. coli* O157:H7.

3. Results

3.1. Impact of spinach leaf-associated microorganisms on the proliferation of E. coli O157:H7 in spinach wash water (SWW)

To assess the impact of spinach indigenous microflora on the proliferation of *E. coli* O157:H7 in SWW, we compared the population sizes of strain MQC57 inoculated in sterile SWW with those in non-sterile SWW during a 10-day incubation at temperatures that are relevant to produce production, processing, and storage. At 4 °C, a small increase in pathogen population was observed in both sterile and non-sterile SWW. The MQC57 population increased 6.1 and 3.1 fold after one-day incubation and decreased to 2.5 and 2.0 fold of initial inoculum on day 10 in sterile and non-sterile SWW, respectively (Fig. 1A). There were no significant differences in growth between MQC57 inoculated in sterile and non-sterile SWW on any of the sampling days (Two-way ANOVA, Sidak's multiple comparisons test, $P > 0.05$). In fact, at 4 °C, neither microflora nor incubation time played a significant role in proliferation of MQC57 (Table 2).

A better growth of MQC57 was observed when incubation temperature was increased from 4 °C to 10 °C. Growth of MQC57 was notable on day 1 in both sterile and non-sterile SWW (Fig. 1B). The largest population size of MQC57 was observed on day 7, with a 16.5-fold and 4.5-fold increase in sterile and non-sterile SWW, respectively. Although there were no significant differences in the population of MQC57 between cells inoculated in sterile and non-sterile SWW on any of the sampling days, a Two-way ANOVA test suggested that spinach microflora impacted the growth of MQC57 significantly in an incubation time-independent manner (Table 2, 10 °C).

The greatest increase in population of MQC57 was observed for cells incubated at 26 °C. In sterile SWW, a 60.6-fold and 87.8-fold increase was detected on day 4 and 7, respectively, which were all significantly larger than the inoculated pathogen population (Unpaired t-test, $P < 0.05$) (Fig. 1C). Although the growth of MQC57 in non-sterile SWW appeared to be slower than that in sterile SWW, a significant increase in MQC57 population was detected on day 2 and day 4 when compared with initial inoculum (Unpaired t-test, $P < 0.05$). A Two-way ANOVA test indicated that, at 26 °C, spinach microflora inhibited the growth of MQC57 in an incubation time-dependent manner (Table 2, 26 °C). The population size of MQC57 in non-sterile SWW was significantly lower than that of the corresponding sample in sterile SWW on day 4 and 7 (Two-way ANOVA, Sidak's multiple comparisons test, $P = 0.04$ for day 4; $P = 0.0009$ for day 7) (Fig. 1C).

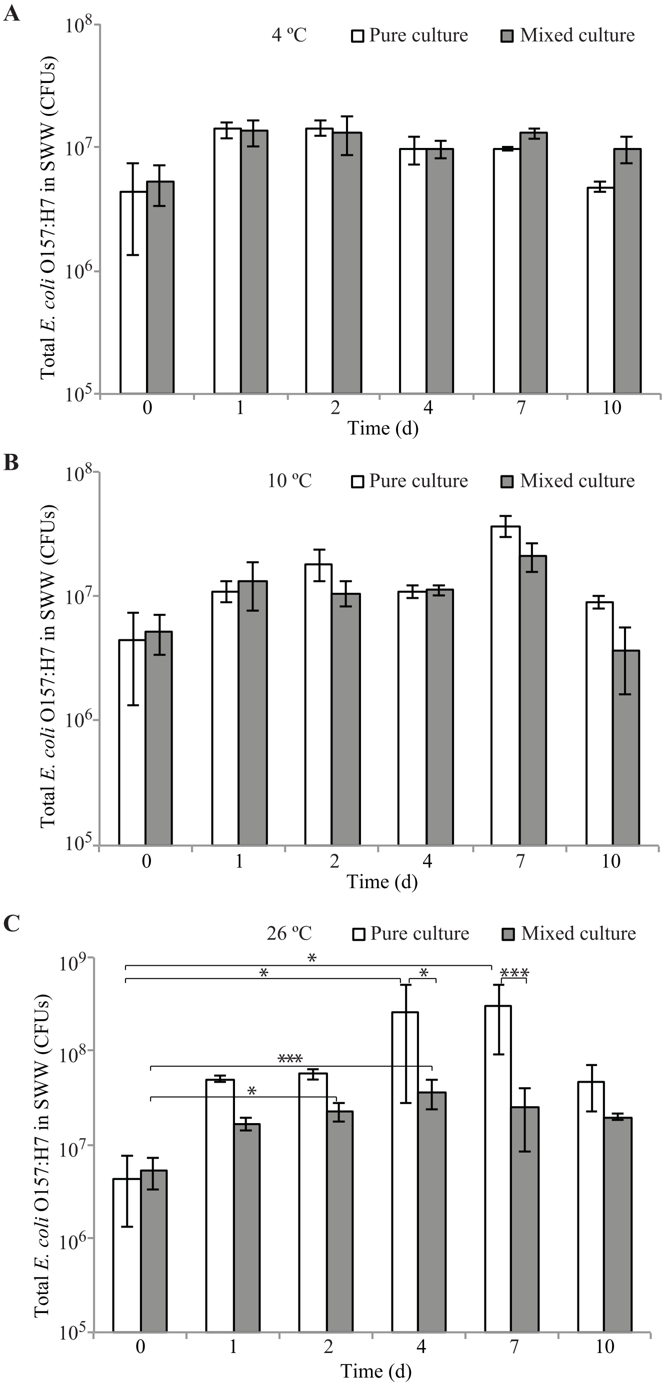

Fig. 1. Population dynamics of *E. coli* O157:H7 in spinach leaf wash water. The data represent the average population size (CFUs) of *E. coli* O157:H7 strain MQC57 inoculated in sterile and non-sterile spinach wash water (SWW) and incubated at 4 °C (**A**), 10 °C (**B**), and 26 °C (**C**) for 10 days. The error bars represent the standard deviations. The significance was indicated by the *P* value of unpaired t-test for same strain comparison or by the *P* value of Two-way ANOVA followed by Sidak's multiple comparisons test for strain comparison in Prism 7 as detailed in the Material and Methods section (*, $P < 0.05$; ***, $P < 0.001$).

3.2. Impact of spinach leaf-associated indigenous microorganisms on E. coli O157:H7 population in mixed biofilm

Since curli fimbriae are known to play a role in cell-to-cell interaction and cell-to-abiotic surface interaction, we included one natural curli-deficient strain (MQC43) and one natural curli-expressing strain

Table 2
Impact of spinach indigenous microflora and incubation time on the proliferation of *E. coli* O157:H7 in spinach wash water at various temperatures.[a]

Factors	4 °C		10 °C		26 °C	
	% of total variation	*P* value	% of total variation	*P* value	% of total variation	*P* value
Interaction	3.6	0.9445	8.8	0.6051	19.4	**0.0410**
Incubation time	14.3	0.4780	22.5	0.1337	23.3	**0.0201**
Microflora	8.35	0.1124	11.5	**0.0384**	24.3	**0.0004**

[a] The impact of microflora and incubation time on proliferation of *E. coli* O157:H7 was evaluated by the *P* value of the Two-way Analysis of Variance (ANOVA). Numbers in bold indicate a *P* value of less than 0.05. A *P* value of the interaction less than 0.05 suggests that the impact of microflora on proliferation of *E. coli* O157:H7 depends on the incubation time.

Table 3
Impact of spinach indigenous microflora and incubation time on *E. coli* O157:H7 mixed biofilm formation at 26 °C.[a]

Factors	*E. coli* O157:H7 strain MQC43		*E. coli* O157:H7 strain MQC57	
	% of total variation	*P* value	% of total variation	*P* value
Interaction	15.0	**0.0128**	8.0	0.3251
Incubation time	11.6	**0.0358**	40.0	**0.0010**
Microflora	43.9	**< 0.0001**	2.6	0.2149

[a] The impact of microflora and incubation time on the mixed biofilm formation by *E. coli* O157:H7 was evaluated by the *P* value of the Two-way Analysis of Variance (ANOVA). Numbers in bold indicate a *P* value of less than 0.05. A *P* value of the interaction less than 0.05 suggests that the impact of microflora on proliferation of *E. coli* O157:H7 depends on the incubation time.

(MQC57) in this assay (Table 1). The curli fimbriae were detected in MQC57 but not in MQC43 under the growing condition.

At 4 °C, biofilm population of MQC43 was in a range of 0.2–0.7% of the total population during the 10-day incubation in sterile SWW, and about 0.1–0.4% of the total population in non-sterile SWW (Fig. 2A). No significant differences in biofilm population were detected between MQC43 cells inoculated in sterile and non-sterile SWW on any of the sampling days (Two-way ANOVA, Sidak's multiple comparisons test, $P > 0.05$). A similar trend was observed for biofilm population of MQC43 when cells were incubated at 10 °C (Fig. 2B). Unlike biofilms at 4 °C or 10 °C, the biofilm population of MQC43 grown in sterile SWW increased greatly during the 10-day incubation and was much larger than the corresponding non-sterile SWW sample when cells were incubated at 26 °C (Fig. 2C). On day 4, 7, and 10, the biofilm population of MQC43 in sterile SWW was significantly larger than that of day 1 (Unpaired t-test, $P < 0.05$) and than that of the corresponding one in non-sterile SWW (Multiple t tests, adjust $P < 0.05$), indicating that strain MQC43 was impaired in participating the mixed biofilm development (Fig. 2C). A Two-way ANOVA test confirmed that spinach microflora impacted the biofilm population of strain MQC43 significantly at 26 °C, and this impact varied at different incubation times (Table 3).

The dynamics of biofilm population of curli-expressing strain MQC57 during the 10-day incubation were similar to that of MQC43 for cells incubated at 4 °C (Figs. 2D) and 10 °C (Fig. 2E). However, unlike strain MQC43, strain MQC57 was proficient in developing mixed biofilm on SSC. The population of MQC57 in mixed biofilm was

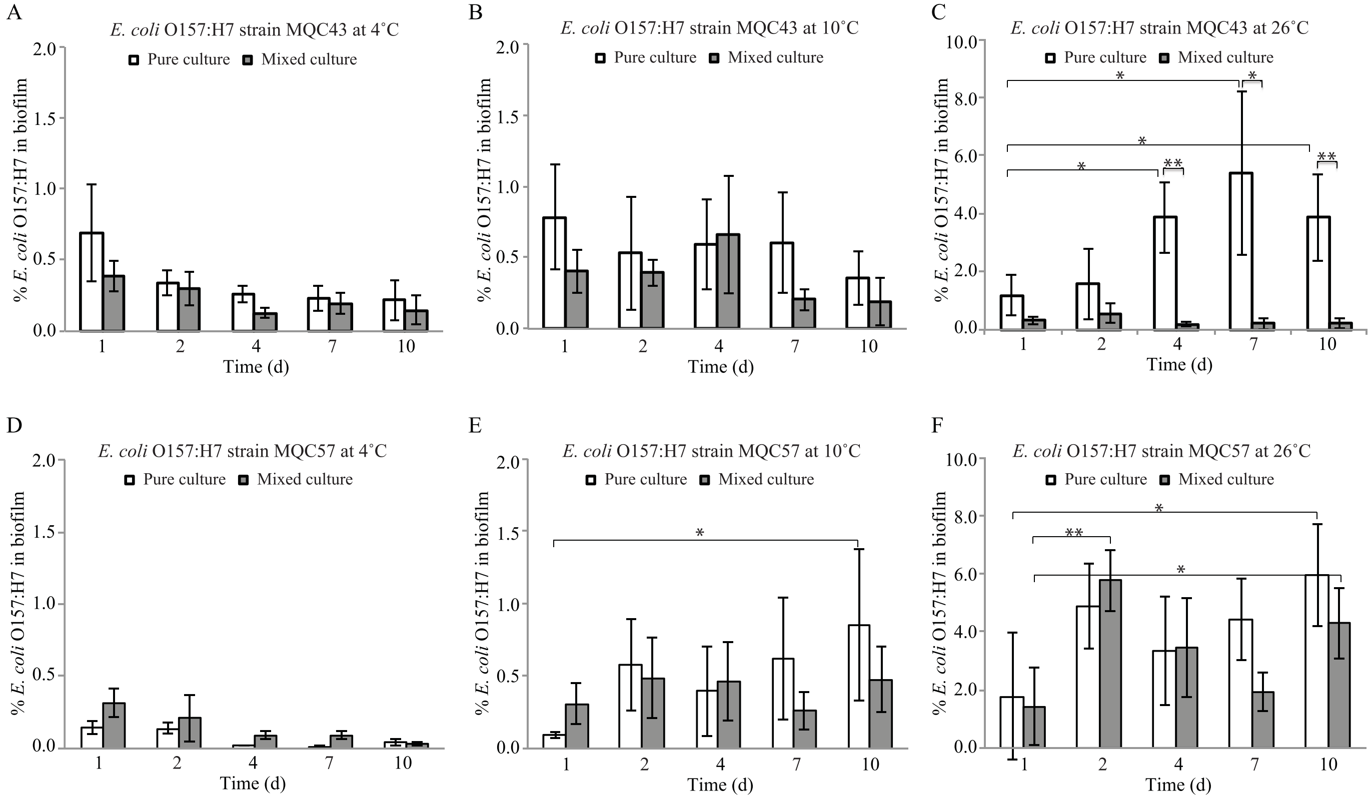

Fig. 2. Biofilms of *E. coli* O157:H7 in spinach leaf wash water. The biofilm population sizes of *E. coli* O157:H7 strain MQC43, a natural curli-deficient variant (**A**, **B**, and **C**), and of strain MQC57, a natural curli-expressing variant (**D**, **E**, and **F**), on the surfaces of stainless steel were determined as described in the Material and Methods section. The data represent the average biofilm population (pathogen population recovered from SSC coupon) expressed as the percent of the initial inoculum. At least four biological replicates were performed for each group. The differences in biofilm population of the same strain during the 10-day incubation were indicated by the *P* value of unpaired t-test; the difference in biofilm population between the pure culture (inoculated in sterile SWW) and the mixed culture (inoculated in non-sterile SWW) were determined by the *P* value of Two-way ANOVA followed by Sidak's multiple comparisons test in Prism 7 as detailed in Material and Methods section (*, $P < 0.05$; **, $P < 0.01$).

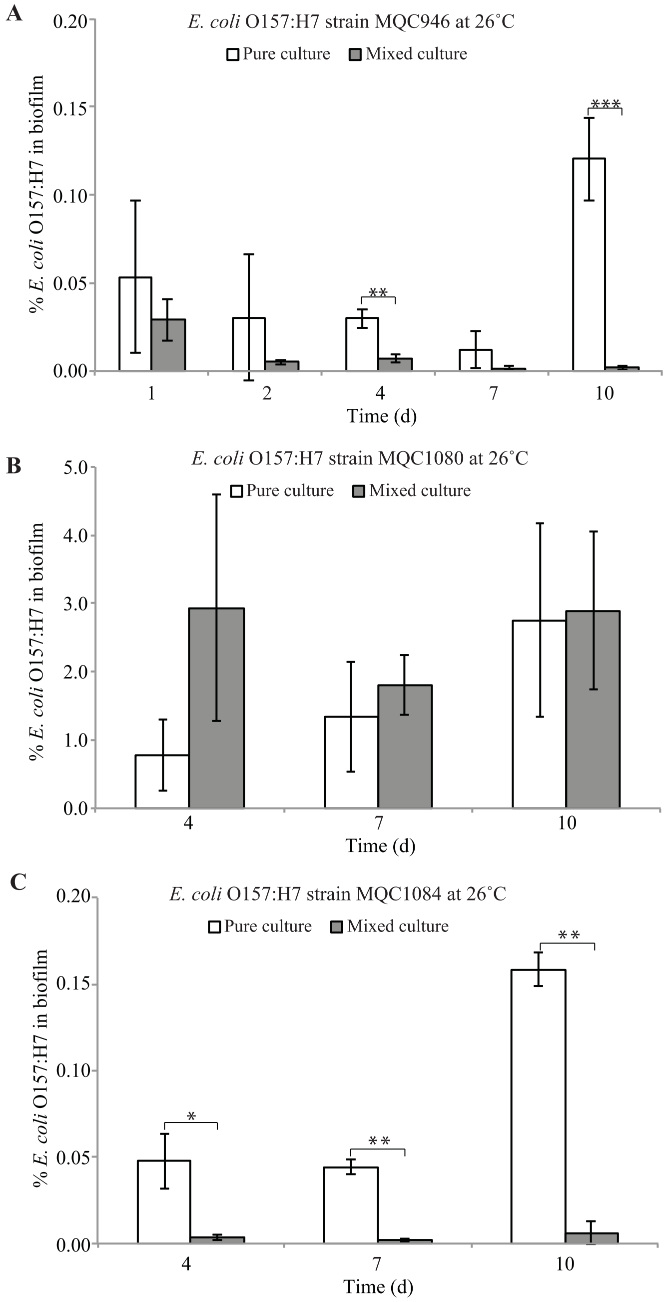

Fig. 3. The contribution of curli fimbriae in the formation of mixed biofilm by *E. coli* O157:H7 and spinach leaf-associated microorganisms on SSC. The biofilm population sizes of *E. coli* O157:H7 *csgA* deletion mutant (MQC946) (**A**), the mutant strain transformed with cloned *csgBAC* operon (MQC1080) (**B**), and the mutant strain transformed with empty expression vector pBBRMCS (MQC1084) (**C**), on SSC were determined as described in the Material and Methods section. The data represent the average cell population recovered from SSC coupon expressed as the percent of the corresponding initial inoculum. At least four biological replicates were performed for each group. The difference in biofilm population between the pure culture (inoculated in sterile SWW) and the mixed culture (inoculated in non-sterile SWW) were determined by the P value of multiple t-test followed by Holm-Sidak method to correct for multiple comparisons using Prism 7 as detailed in the Material and Methods section (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

significantly larger than that of MQC43 on day 2 (10.1-fold), day 4 (17.0-fold), day 7 (8.9-fold), and day 10 (18.9-fold) at 26 °C (Fig. 2C and F, Mixed culture) (Multiple t tests, adjust $P < 0.001$ for day 2; $P = 0.003$ for day 4; $P = 0.015$ for day 7; and $P = 0.005$ for day 10). The population of strain MQC57 in mixed biofilm was at a level similar to that of MQC57 in pure culture on all sampling days (Two-way ANOVA, Sidak's multiple comparisons test, $P > 0.05$). Under both growth conditions, a small but significant increase in biofilm population of MQC57 was detected on day 10 compared with day 1 (Unpaired t-test, $P < 0.05$) (Fig. 2F). Furthermore, unlike strain MQC43, Two-way ANOVA test suggested that only incubation time was a major factor impacting the biofilm formation of MQC57 in SWW (Table 3).

3.3. Curli-mutant was impaired in formation of mixed biofilm on SSC

To confirm that the deficiency of strain MQC43 in participating mixed biofilm formation was due to lack of curli fimbriae, we generated a curli-deletion mutant in strain MQC17, the parental strain of MQC57, and compared the biofilm population of mutant strain in the presence (mixed biofilm) and absence of spinach leaf-associated microorganisms (Fig. 3A). Although deletion of curli in MQC17 did not completely abolish its biofilm formation on SSC (either pure or mixed biofilm), the biofilm populations of mutant strain (MQC946) decreased greatly and significantly compared with curli-expressing strain MQC57. In sterile SWW, there was 160-, 112-, 361-, and 49-fold decrease on day 2, 4, 7, and 10, respectively (Two-way ANOVA, Sidak's multiple comparisons test, $P < 0.0001$ for day 2; $P = 0.0036$ for day 4; $P = 0.0001$ for day 7; and $P < 0.0001$ for day 10) (Figs. 2F and 3A, Pure culture). In non-sterile SWW, there was 1134-, 479-, 1254-, and 2346-fold decrease on day 2, 4, 7, and 10, respectively (Two-way ANOVA, Sidak's multiple comparisons test, $P < 0.0001$ for day 2 and day 4; $P = 0.0207$ for day 7; and $P < 0.0001$ for day 10) (Figs. 2F and 3A, Mixed culture). Furthermore, similar to strain MQC43, the population of strain MQC946 in mixed biofilm dropped significantly compared with that of the pure biofilm at the late stage of incubation with a 4.1-, 8.0-, and 65.9-fold decrease on day 4, 7, and 10, respectively (Multiple t tests, adjust $P = 0.0021$ for day 4; $P = 0.0006$ for day 10) (Fig. 3A).

3.4. Expression of curli in E. coli O157:H7 restored the pathogen population in mixed biofilm

We next examined the biofilm population of curli-deletion mutant MQC946 when curli genes were expressed *in trans*. In sterile SWW, biofilm population of strain MQC1080 (MQC946 transformed with cloned curli genes) was 13.5, 22.1, and 25.3 fold of strain MQC1084 (MQC946 transformed with empty expression vector) on day 4, 7, and 10, respectively (Fig. 3B and C, Pure culture). Similarly, the population of strain MQC1080 in mixed biofilm was 833.6, 903.8, and 462.0 fold of the strain MQC1084 on day 4, 7, and 10, respectively (Fig. 3B and C, Mixed culture). In both culture conditions, the differences in either pure or mixed biofilm populations between control strain (MQC1084) and the curli-complementary strain MQC1080 were significant on all sampling days (Multiple t tests, adjust $P < 0.05$). Similar to MQC946, the control strain MQC1084 was deficient in participating in mixed biofilm formation. On all sampling days, the population of strain MQC1084 in mixed biofilm was significantly lower than that in pure biofilm (Multiple t tests, adjust $P = 0.035$ for day 4; $P = 0.002$ for day 7 and day 10) (Fig. 3C); however, this deficiency was rescued once the biogenesis of curli was restored in strain MQC946 (Fig. 3B).

4. Discussion

Knowledge about the persistence of STEC in field production and in postharvest processing environments is highly desired as the occurrence of outbreaks of STEC infections linked to leafy vegetables is persisting. Minimally processed fresh produce including precut vegetables is one of the fastest growing fields in the produce industry due to increasing consumer demand for fresh, healthy, and convenient foods (Gil et al., 2015). However, mechanical harvesting and processing of leafy vegetables is likely to injure plant tissue and cause leakage of cellular contents onto tools and equipment. We previously reported that STEC O157:H7 multiplied rapidly in fresh spinach lysates with a growth rate much faster than those of native phyllosphere colonists (Carter et al., 2012). A similar behavior was observed for STEC O157:H7 inoculated in spinach leaf wash water. Growth of the inoculated pathogen was observed at cold and room temperatures, suggesting a great ability of *E. coli* O157:H7 in utilizing plant-derived substrates, even at very low concentrations, similarly to a recent report on the enhanced growth of *Salmonella* in salad juices at refrigeration temperatures (Koukkidis et al., 2017). Proliferation of STEC in spinach wash water implies a potential amplification of this pathogen in fields and at cold storage and processing temperatures and emphasizes the need to develop effective control to reduce the risk of pathogen contamination.

Curli are known to mediate direct contact between bacterial cells and abiotic surfaces and play an essential role in biofilm maturation (Brandl et al., 2011; Carter et al., 2016; DeBenedictis et al., 2016). However, our results indicated that contribution of curli fimbriae to biofilm formation is strain-dependent in STEC, since, in pure culture, the biofilm populations of STEC strain MQC43, a natural curli-deficient variant, were comparable to that of strain MQC57, a natural curli-expressing variant under the conditions tested. Deletion of curli genes in strain MQC17 impaired the biofilm formation of this strain largely, suggesting that curli fimbriae indeed enhance the development of biofilm, similarly to a previous report on biofilm formation of other *E. coli* O157:H7 strains on SSC (Ryu et al., 2004). However, the success of strain MQC43 in developing pure biofilm on SSC suggests a curli-independent mechanism present in STEC O157:H7 for attachment to and establishment of biofilm.

The vast difference in STEC biofilm population between the curli-positive and curli-negative strains indicates that curli fimbriae confer STEC a competitive trait by promoting interactions of this human pathogen with the plant microflora. Such interactions not only enhance the environmental survival of STEC as has been suggested in several studies, but also expand the ecological niches of STEC (Giaouris et al., 2015; Poza-Carrion et al., 2013; Schwering et al., 2013; Wang et al., 2013). Many epiphytic bacteria, including *Pseudomonas* spp., are proficient in biofilm formation. The synergistic interaction between *E. coli* and *P. putida* facilitated surface attachment (Castonguay et al., 2006), and enhanced *E. coli* O157 biofilm formation by *P. aeruginosa*, an ubiquitous environmental bacterium (Klayman et al., 2009), and by bacteria that are native to fresh produce processing environments (Liu et al., 2014). Such interactions would introduce STEC to and promote the colonization of STEC at niches that are normally hostile and uninhabitable by STEC themselves.

5. Conclusions

We investigated environmental and bacterial factors that may contribute to the biofilm formation of enteric pathogen *E. coli* O157:H7 on surfaces of stainless steel, a common material used in the food industry. We first examined the population dynamics of *E. coli* O157:H7 in spinach leaf washes and observed a proliferation of the inoculated pathogen at 4 °C, 10 °C, and 26 °C, implying a potential of amplification in pathogen population even under cold storage conditions. We next examined the biofilm population of *E. coli* O157:H7 in the presence and absence of spinach-associated microorganisms, and discovered a distinct difference between the curli-expressing and curli-deficient strains. Curli-deficient strain was impaired in participating in mixed biofilm formation by *E. coli* O157:H7 and spinach-associated microorganisms; whereas this deficiency was rescued when curli biogenesis was restored. Our data suggest that curli fimbriae not only promote the direct contact between pathogen cells and stainless steel surfaces, but also mediate interactions between pathogen cells and spinach-associated microorganisms. Such interactions would expand the ecological niches of *E. coli* O157:H7 and enhance the persistence of this pathogen in produce field production and postharvest processing environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.024.

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