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Dual – Species Biofilm Formation by Shiga – toxin Producing *Escherichia coli* O157:H7 and O26:H11 serotypes

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Abstract

E. coli O26:H11 strains could outgrow O157:H7 companion strains in planktonic and biofilm phases, and also effectively compete with pre-colonized O157:H7 cells to establish themselves in mixed biofilms. *E. coli* O157:H7 strains were unable to displace preformed O26:H11 biofilms. Therefore, *E. coli* O26:H11 remains a potential risk in food safety.

Shiga-toxin producing *Escherichia coli* strains (STEC) of various serotypes are important foodborne pathogens responsible for numerous outbreaks with symptoms ranging from bloody diarrhea to other more severe diseases such as hemolytic uremic syndrome (HUS). Of the many STEC serotypes, O157:H7 is the most frequently identified serotype that causes foodborne outbreaks worldwide. Meanwhile, six non-O157 STEC serotypes, including O26, O45, O103, O111, O121 and O145, have attracted significant attention from the scientific and regulatory fields lately since they have been increasingly associated with serious outbreaks. It was reported that these six STEC serotypes, now referred to as "the big six", were responsible for approximately 70% of all non-O157 STEC infections from 1983 to 2002 (*4*). In 2010, these non-O157 STEC serotypes collectively caused more human infections in the U.S. than STEC O157:H7. The Food Safety and Inspection Service (FSIS) published a Federal Register notice in September 2011 announcing their intent to regulate these non-O157 STECs as adulterants in certain raw beef products (*11*). Among "the big six", O26 is the most common non-O157 STEC isolated from specimens submitted to the Centers for Disease Control and Prevention (CDC) for serotyping. Recovery of STEC O26 from cattle and beef products has been reported in different countries (*1, 3, 8, 13*). In particular, serotype O26:H11 has been regarded as one of the most dangerous non-O157 STEC due to its enhanced virulence and ability to cause diarrhea and HUS (*2, 3*). The recent FSIS recall of 8500 pounds of Cargill ground beef contaminated by STEC O26:H11 was associated with foodborne outbreaks in Maine and New York. These outbreaks highlight the critical needs to prevent the transmission and food contamination by these pathogens.

Biofilm formation is one of the major strategies that support bacterial survival under adverse circumstances. In nature, bacteria are able to form single – species biofilms, or coexist in

multi-species communities and form mixed biofilms on a wide variety of solid surfaces. In the food industry, attachment of foodborne pathogens on food products and contact surfaces can be enhanced by biofilm formation, and the detached biofilms could become a continuous source of cross – contamination. It has been shown that multiple bacterial species, including *E. coli*, *Salmonella, Staphylococcus, Bacillus*, and *Pseudomonas,* etc, could coexist and form biofilms in meat processing plants (*7*). Studies focusing on STEC O157:H7 have shown that surface attachment of an O157:H7 strain unable to form single – species biofilm was enhanced by forming mixed biofilms with an *E. coli* O-:H4 companion strain (*10*). Similarly, pre-formed *Acinetobacter calcoaceticus* biofilms enhanced O157:H7 colonization on solid surfaces under both static and dynamic growth conditions (*6*). However, an area not well explored is the non-O157 STEC biofilms and the effects of coexistence of STEC O157 and non-O157 serotypes on dual-species biofilm formation. The interactions between the species could profoundly affect biofilm development, composition and structure. Research in this area should be of great interest from a food safety standpoint because coexistence of multiple bacterial species is frequently observed in food processing plants, and multispecies biofilms often demonstrate higher resistance to common sanitizers compared to single-species biofilms or the corresponding planktonic cells (*10*). Therefore, the objective of this study was to investigate how coexistence of STEC O157:H7 and O26:H11 serotypes would affect bacterial growth, colonization, and the mixed biofilm composition.

Two STEC O157:H7 strains and three O26:H11 strains that exhibited curli expression at different levels and demonstrated various biofilm forming ability on polystyrene surfaces in our previous study (*12*) were selected for the investigation (Table 1). We first compared the growth capability of these STEC strains while they replicated individually. Each strain was grown

82 statically in Lennox Broth without salt (LB-NS medium) at room temperature (22 – 25°C), the
83 same experimental condition as used in the biofilm assays. Bacterial growth was examined after
84 24, 48, and 72 hours by diluting and plating individual cultures onto Tryptic Soy Agar (Difco,
85 Beckton Dickinson, Sparks, MD) plates or ChromAgar O157 agar (DRG International Inc.,
86 Mountainside, NJ) plates supplemented with 5 mg/L novobiocin and 2.5 mg/L tellurite (*Sigma,*
87 St. Louis, MO) (ntChromAgar). On each type of the agar plate, no significant difference in
88 bacterial growth as measured by colony numeration was observed among the five STEC strains
89 (data not shown).
90 Since multiple bacterial species often coexist, compete, and colonize on solid surfaces in
91 food processing environments, we investigated how coexistence of these two serotypes would
92 affect bacterial planktonic growth and mixed biofilm formation. Equal numbers of bacteria (≈ 1
93 x 10^6 CFU) from one O157:H7 strain and one O26:H11 strain were mixed to make dual-strain
94 cultures. The cultures were grown statically on 96-well polystyrene plates at room temperature
95 for 72 hours. Bacterial cells in planktonic suspensions and in biofilms were harvested as
96 previously described (*12*), serially diluted in LB-NS broth and plated onto ntChromAgar plates
97 for colony enumeration after overnight incubation at 37°C. The two serotypes were distinguished
98 by colony morphology as the O157:H7 and O26:H11 strains would form pink and blue colonies
99 on the plates, respectively. Substantially higher cell numbers of O26:H11 compared to O157:H7
100 strains were observed in all dual-species planktonic suspensions (Table 2), indicating that the
101 O26:H11 strains were able to effectively compete with and outgrow O157:H7 strains in co-
102 inoculated suspensions even though the two serotypes reached similar cell densities while
103 cultured individually for 72 hours. Similarly, of the strain-pairs tested, the percentages of
104 O26:H11 cells comprising the 72-h mixed biofilms also were consistently higher than that of the

O157:H7 companion strains. These data indicated that the O26:H11 strains could successfully outcompete O157:H7 strains during the biofilm forming process as well, thus, maintain a higher population density and become the major component of the mixed biofilms. Although the percentages of the O26:H11 cells in the mixtures varied among the different strain pairs, there was a clear trend of serotype O26:H11 being the majority in mixed biofilms and in medium suspension. This suggests that the ability of serotype O26:H11 to outcompete serotype O157:H7 during planktonic growth and biofilm formation may be serotype-related rather than strain-specific. To further confirm these observations, indirect enzyme-linked immunosorbent assays (ELISA) were performed as previously described using monoclonal anti-O157 or anti-O26 antibodies (*9, 14*) to measure each serotype in the mixtures. The ELISA data were in consistent with the colony enumeration results, indicating that the O26:H11 strains were the predominant species within the mixtures (data not shown).

To further explore how bacteria would colonize and form biofilms on the solid surface with a preformed biofilm by other species, which represents a likely event in commercial meat plants, pure cultures of O157:H7 or O26:H11 strains were allowed to form single-species biofilms on 96-well plates for 72 hours as described above. After removal of supernatants and the loosely attached cells with washing, overnight cultures of the other serotype were diluted and added to the plates to allow mixed planktonic growth and biofilm formation for another 72 h in the presence of the pre-existing biofilms. At the end of the incubation period, samples were harvested and processed as described above.

We first tested the development of O157:H7 biofilm on an established O26:H11 biofilm. In all cases the O26:H11 cells were present in substantially higher numbers compared to O157:H7 cells at 72 h following O157:H7 inoculation, occupying over 90% of the total populations in the

mixed biofilms as well as in planktonic growth (Table 2). These observations indicated that in the presence of the O26:H11 biofilms, the O157:H7 strains were unable to grow efficiently and compete with the O26:H11 strains under heterologous culture conditions to displace the pre-existing O26:H11 biofilms. With the co-inoculated or pre-colonized O26:H11 companion strains, the fact that O157:H7 strain FSIS62 was not detected in the suspensions but was present in the mixed biofilms suggested that biofilm formation could offer protection and enhance O157:H7 cell survival during competition with companion strains.

In additional experiments, each O26:H11 strain was inoculated onto an O157:H7 biofilm that had been developed for 72 h. After incubation for an additional 72 h, all three O26:H11 strains successfully outgrew O157:H7 strain FSIS62 in planktonic suspensions (>80%). When cultured with biofilms by O157:H7 strain FSIS11, O26:H11 strain DEC 10B outgrew strain FSIS11 in the planktonic phase (≈ 75%), and the other two O26:H11 strains each occupied approximately 20% of the cell populations in the suspensions. Therefore, the pre-formed O157:H7 biofilms did not inhibit cell growth of the O26:H11 strains. Meanwhile, biofilm measurements indicated that the O26:H11 strains in all strain pairs except one (FSIS62/7-14 50A) were able to establish themselves in the mixed biofilms after 72 h incubation in the presence of the preformed O157:H7 biofilm. The FSIS62/7-14 50A strain pair did not have a low percentage of the O26:H11 cells in planktonic suspension, indicating that the low amount of O26:H11 cells in the mixed biofilms was not due to cell outgrowth by the pre-colonized O157:H7 cells. However, the percentages of O26:H11 cells in these mixed biofilms were generally lower compared to the dual-species biofilms formed by the two serotypes inoculated simultaneously. These observations suggested that coexistence and growth competition in planktonic phase plays a role in the effective outgrowth of the O26:H11 strains during mixed

biofilm development, while early stage pre-colonization favors O157:H7 cell survival and biofilm maintenance.

Bacterial species could compete over essential nutritional resources, or inhibit the growth of the competitors by producing inhibitory agents. To understand the mechanism for the domination of serotype O26:H11, O26:H11 culture supernatants were filter-sterilized using syringe filters (0.20 µm pore size, Corning, NY) then applied at 20 µL/drop onto the surface of TSA agar plates seeded with 5-h broth culture of the O157:H7 strains, which formed a confluent lawn after overnight incubation. No growth inhibition zone was observed on the plates within the area covered by the filtered O26:H11 supernatants, suggesting that the capability of the O26:H11 strains to outgrow O157:H7 strains was not mediated by the production of inhibitory agents. To further identify the genetic determinants responsible for such dominating capability, future studies would focus on generating O26:H11 mutant strains deficient in certain replication or virulence – associated genes, and comparing the competition results from O157:H7 and O26:H11 mixtures, using either O26:H11 wild-type strains or the mutant strains.

In conclusion, we have shown that STEC O26:H11 strains were able to effectively outgrow O157:H7 stains in both planktonic and biofilm phases during coexistence via simultaneous inoculation. In the presence of pre-formed O157:H7 biofilms, the O26:H11 strains could still compete with the colonized O157:H7 cells and establish themselves in the mixed biofilms, while serotype O157:H7 appeared to be a poor colonizer if O26:H11 cells pre-occupied the solid surfaces. The fact that the O26:H11 strains have the strong ability to outcompete serotype O157:H7 highlights the potential risk of this non-O157 STEC in food safety and calls for increased attention to the non-O157 STEC biofilms in food processing facilities.

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233 **Table 1.** *Bacterial strains, virulence genes, curli expression on Congo Red Indicator plates, and*

234 *measurement of single – species biofilm formation on 96-well polystyrene plates.*

Serotype	Strain	Origin[a]	*stx1*	*stx2*	*eae*	*hlyA*	Curli [b]	Biofilm [c]	Ref
O157:H7	FSIS11	G		y	y	y	-	4.50 ± 0.24	MH[d]
	FSIS62	G	y	y	y	y	±	9.83 ± 1.18	MH[d]
O26:H11	7-14 50A	B		y	y	y	++	13.17 ± 3.06	MARC[e]
	DEC 10B	H	y		y	y	±	9.50 ± 1.18	(*13*)
	DEC 10E	B	y	y	y	y	-	10.83 ± 2.12	(*13*)

235 [a] G: ground beef; B: bovine; H: human.

236 [b] Curli expression was confirmed by streaking the strains onto Congo red indicator agar plates,

237 and the expression level was evaluated based on colony color and morphology on the plates.

238 [c] Each strain was allowed to form biofilms on 96-well polystyrene plates at room temperature for

239 72 hours. Data are shown as mean x 10^6 CFU/well ± SD; n=3.

240 [d] Provided by Dr. Marcus Head, USDA, FSIS.

241 [e] U.S. Meat Animal Research Center strain collection.

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245 **Table 2**. *Bacterial planktonic growth [mean* x *10^7CFU/mL ± SD (% in the mixture); n=3] and*

246 *biofilm formation [mean* x *10^6CFU/well ± SD (% in the mixture); n=3] on 96-well polystyrene*

247 *plates.*

Strain combination	Planktonic (10^7CFU/mL)		Biofilms (10^6CFU/well)	
(O157:H7/O26:H11)	O157:H7	O26:H11	O157:H7	O26:H11
	Co-inoculated			
FSIS11 / 7-14 50A	8.75 ± 1.34 (38.2%)	14.15 ± 1.35 (61.8%)	3.00 ± 0.94 (31.0%)	6.67 ± 0.94 (69.0%)
FSIS11 / DEC 10B	0.15 ± 0.07 (0.5%)	29.30 ± 5.23 (99.5%)	0.83 ± 0.70 (8.2%)	9.33 ± 0.94 (91.8%)
FSIS11 / DEC 10E	1.00 ± 0.14 (3.4%)	28.65 ± 3.04 (96.6%)	0.67 ± 0.47 (7.7%)	8.00 ± 0.10 (92.3%)
FSIS62 / 7-14 50A	< LOD *	13.00 ± 2.97 (100%)	4.67 ± 0.94 (28.6%)	11.67 ± 3.30 (71.4%)
FSIS62 / DEC 10B	< LOD *	28.50 ± 4.95 (100%)	4.67 ± 1.41 (31.1%)	10.33 ± 0.94 (68.9%)
FSIS62 / DEC 10E	< LOD *	31.35 ± 1.77 (100%)	3.67 ± 0.0 (26.5%)	10.17 ± 3.06 (73.5%)
	O26:H11 pre-colonized			
FSIS11 / 7-14 50A	0.30 ± 0.0 (2.3%)	12.75 ± 0.78 (97.7%)	0.38 ± 0.29 (2.3%)	15.58 ± 0.59 (97.7%)
FSIS11 / DEC 10B	0.05 ± 0.07 (0.2%)	25.35 ± 1.20 (99.8%)	0.21 ± 0.18 (3.4%)	5.92 ± 1.30 (96.6%)
FSIS11 / DEC 10E	< LOD *	30.10 ± 3.11 (100%)	0.17 ± 0.11 (2.1%)	7.88 ± 0.53 (97.1%)
FSIS62 / 7-14 50A	< LOD *	17.25 ± 1.06 (100%)	1.42 ± 0.24 (7.3%)	17.92 ± 0.94 (92.7%)
FSIS62 / DEC 10B	< LOD *	23.45 ± 0.21 (100%)	0.63 ± 0.06 (9.5%)	5.96 ± 0.18 (90.5%)
FSIS62 / DEC 10E	< LOD *	19.00 ± 0.57 (100%)	0.38 ± 0.06 (2.9%)	12.42 ± 5.42 (97.1%)
	O157:H7 pre-colonized			
FSIS11 / 7-14 50A	8.90 ± 1.70 (79.1%)	2.35 ± 0.64 (20.9%)	1.58 ± 0.12 (82.6%)	0.33 ± 0.0 (17.4%)
FSIS11 / DEC 10B	7.25 ± 0.64 (25.3%)	21.35 ± 0.07 (74.7%)	1.67 ± 0.71 (50.0%)	1.67 ± 0.94 (50.0%)
FSIS11 / DEC 10E	9.50 ± 1.41 (81.5%)	2.15 ± 0.49 (18.5%)	3.00 ± 0.90 (56.3%)	2.33 ± 1.41 (43.7%)
FSIS62 / 7-14 50A	1.75 ± 0.07 (20.2%)	6.90 ± 0.71 (79.8%)	16.50 ± 1.18 (95.2%)	0.83 ± 0.0 (4.8%)
FSIS62 / DEC 10B	1.20 ± 0.42 (4.2%)	27.40 ± 0.80 (95.8%)	2.75 ± 0.82 (54.1%)	2.33 ± 1.18 (45.9%)
FSIS62 / DEC 10E	2.80 ± 0.14 (19.6%)	11.45 ± 0.92 (80.4%)	1.92 ± 0.59 (37.1%)	3.25 ± 0.35 (62.9%)

248 * < LOD: lower than the limit of detection (1 x 10^5 CFU/mL).

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