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

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1   Running head: Dual-species biofilms by Shiga-toxin producing *E. coli*

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4   O26:H11 serotypes

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16   **Running head:** Dual-species biofilms by Shiga-toxin producing *E. coli*

17   Key words: *Escherichia coli* O157:H7, O26:H11, dual-species biofilm formation

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27   **Abstract**

28   *E. coli* O26:H11 strains could outgrow O157:H7 companion strains in planktonic and

29   biofilm phases, and also effectively compete with pre-colonized O157:H7 cells to establish

30   themselves in mixed biofilms. *E. coli* O157:H7 strains were unable to displace preformed

31   O26:H11 biofilms. Therefore, *E. coli* O26:H11 remains a potential risk in food safety.

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36   Shiga-toxin producing *Escherichia coli* strains (STEC) of various serotypes are important

37 foodborne pathogens responsible for numerous outbreaks with symptoms ranging from bloody

38 diarrhea to other more severe diseases such as hemolytic uremic syndrome (HUS). Of the many

39 STEC serotypes, O157:H7 is the most frequently identified serotype that causes foodborne

40 outbreaks worldwide. Meanwhile, six non-O157 STEC serotypes, including O26, O45, O103,

41 O111, O121 and O145, have attracted significant attention from the scientific and regulatory

42 fields lately since they have been increasingly associated with serious outbreaks. It was reported

43 that these six STEC serotypes, now referred to as "the big six", were responsible for

44 approximately 70% of all non-O157 STEC infections from 1983 to 2002 (*4*). In 2010, these non-

45 O157 STEC serotypes collectively caused more human infections in the U.S. than STEC

46 O157:H7. The Food Safety and Inspection Service (FSIS) published a Federal Register notice in

47 September 2011 announcing their intent to regulate these non-O157 STECs as adulterants in

48 certain raw beef products (*11*). Among "the big six", O26 is the most common non-O157 STEC

49 isolated from specimens submitted to the Centers for Disease Control and Prevention (CDC) for

50 serotyping. Recovery of STEC O26 from cattle and beef products has been reported in different

51 countries (*1, 3, 8, 13*). In particular, serotype O26:H11 has been regarded as one of the most

52 dangerous non-O157 STEC due to its enhanced virulence and ability to cause diarrhea and HUS

53 (*2, 3*). The recent FSIS recall of 8500 pounds of Cargill ground beef contaminated by STEC

54 O26:H11 was associated with foodborne outbreaks in Maine and New York. These outbreaks

55 highlight the critical needs to prevent the transmission and food contamination by these

56 pathogens.

57   Biofilm formation is one of the major strategies that support bacterial survival under

58 adverse circumstances. In nature, bacteria are able to form single – species biofilms, or coexist in

59   multi-species communities and form mixed biofilms on a wide variety of solid surfaces. In the

60   food industry, attachment of foodborne pathogens on food products and contact surfaces can be

61   enhanced by biofilm formation, and the detached biofilms could become a continuous source of

62   cross – contamination. It has been shown that multiple bacterial species, including *E. coli*,

63   *Salmonella, Staphylococcus, Bacillus*, and *Pseudomonas,* etc, could coexist and form biofilms in

64   meat processing plants (*7*). Studies focusing on STEC O157:H7 have shown that surface

65   attachment of an O157:H7 strain unable to form single – species biofilm was enhanced by

66   forming mixed biofilms with an *E. coli* O-:H4 companion strain (*10*). Similarly, pre-formed

67   *Acinetobacter calcoaceticus* biofilms enhanced O157:H7 colonization on solid surfaces under

68   both static and dynamic growth conditions (*6*). However, an area not well explored is the non-

69   O157 STEC biofilms and the effects of coexistence of STEC O157 and non-O157 serotypes on

70   dual-species biofilm formation. The interactions between the species could profoundly affect

71   biofilm development, composition and structure. Research in this area should be of great interest

72   from a food safety standpoint because coexistence of multiple bacterial species is frequently

73   observed in food processing plants, and multispecies biofilms often demonstrate higher

74   resistance to common sanitizers compared to single-species biofilms or the corresponding

75   planktonic cells (*10*). Therefore, the objective of this study was to investigate how coexistence of

76   STEC O157:H7 and O26:H11 serotypes would affect bacterial growth, colonization, and the

77   mixed biofilm composition.

78   Two STEC O157:H7 strains and three O26:H11 strains that exhibited curli expression at

79   different levels and demonstrated various biofilm forming ability on polystyrene surfaces in our

80   previous study (*12*) were selected for the investigation (Table 1). We first compared the growth

81   capability of these STEC strains while they replicated individually. Each strain was grown

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82   statically in Lennox Broth without salt (LB-NS medium) at room temperature ($22 - 25^{o}$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 ($\approx$ 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

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105   O157:H7 companion strains. These data indicated that the O26:H11 strains could successfully

106   outcompete O157:H7 strains during the biofilm forming process as well, thus, maintain a higher

107   population density and become the major component of the mixed biofilms. Although the

108   percentages of the O26:H11 cells in the mixtures varied among the different strain pairs, there

109   was a clear trend of serotype O26:H11 being the majority in mixed biofilms and in medium

110   suspension. This suggests that the ability of serotype O26:H11 to outcompete serotype O157:H7

111   during planktonic growth and biofilm formation may be serotype-related rather than strain-

112   specific. To further confirm these observations, indirect enzyme-linked immunosorbent assays

113   (ELISA) were performed as previously described using monoclonal anti-O157 or anti-O26

114   antibodies (*9, 14*) to measure each serotype in the mixtures. The ELISA data were in consistent

115   with the colony enumeration results, indicating that the O26:H11 strains were the predominant

116   species within the mixtures (data not shown).

117   To further explore how bacteria would colonize and form biofilms on the solid surface with

118   a preformed biofilm by other species, which represents a likely event in commercial meat plants,

119   pure cultures of O157:H7 or O26:H11 strains were allowed to form single-species biofilms on

120   96-well plates for 72 hours as described above. After removal of supernatants and the loosely

121   attached cells with washing, overnight cultures of the other serotype were diluted and added to

122   the plates to allow mixed planktonic growth and biofilm formation for another 72 h in the

123   presence of the pre-existing biofilms. At the end of the incubation period, samples were

124   harvested and processed as described above.

125   We first tested the development of O157:H7 biofilm on an established O26:H11 biofilm. In

126   all cases the O26:H11 cells were present in substantially higher numbers compared to O157:H7

127   cells at 72 h following O157:H7 inoculation, occupying over 90% of the total populations in the

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128 mixed biofilms as well as in planktonic growth (Table 2). These observations indicated that in

129 the presence of the O26:H11 biofilms, the O157:H7 strains were unable to grow efficiently and

130 compete with the O26:H11 strains under heterologous culture conditions to displace the pre-

131 existing O26:H11 biofilms. With the co-inoculated or pre-colonized O26:H11 companion strains,

132 the fact that O157:H7 strain FSIS62 was not detected in the suspensions but was present in the

133 mixed biofilms suggested that biofilm formation could offer protection and enhance O157:H7

134 cell survival during competition with companion strains.

135   In additional experiments, each O26:H11 strain was inoculated onto an O157:H7 biofilm

136 that had been developed for 72 h. After incubation for an additional 72 h, all three O26:H11

137 strains successfully outgrew O157:H7 strain FSIS62 in planktonic suspensions (>80%). When

138 cultured with biofilms by O157:H7 strain FSIS11, O26:H11 strain DEC 10B outgrew strain

139 FSIS11 in the planktonic phase ($\approx$ 75%), and the other two O26:H11 strains each occupied

140 approximately 20% of the cell populations in the suspensions. Therefore, the pre-formed

141 O157:H7 biofilms did not inhibit cell growth of the O26:H11 strains. Meanwhile, biofilm

142 measurements indicated that the O26:H11 strains in all strain pairs except one (FSIS62/7-14

143 50A) were able to establish themselves in the mixed biofilms after 72 h incubation in the

144 presence of the preformed O157:H7 biofilm. The FSIS62/7-14 50A strain pair did not have a low

145 percentage of the O26:H11 cells in planktonic suspension, indicating that the low amount of

146 O26:H11 cells in the mixed biofilms was not due to cell outgrowth by the pre-colonized

147 O157:H7 cells. However, the percentages of O26:H11 cells in these mixed biofilms were

148 generally lower compared to the dual-species biofilms formed by the two serotypes inoculated

149 simultaneously. These observations suggested that coexistence and growth competition in

150 planktonic phase plays a role in the effective outgrowth of the O26:H11 strains during mixed

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

152 biofilm maintenance.

153   Bacterial species could compete over essential nutritional resources, or inhibit the growth

154 of the competitors by producing inhibitory agents. To understand the mechanism for the

155 domination of serotype O26:H11, O26:H11 culture supernatants were filter-sterilized using

156 syringe filters (0.20 µm pore size, Corning, NY) then applied at 20 µL/drop onto the surface of

157 TSA agar plates seeded with 5-h broth culture of the O157:H7 strains, which formed a confluent

158 lawn after overnight incubation. No growth inhibition zone was observed on the plates within the

159 area covered by the filtered O26:H11 supernatants, suggesting that the capability of the O26:H11

160 strains to outgrow O157:H7 strains was not mediated by the production of inhibitory agents. To

161 further identify the genetic determinants responsible for such dominating capability, future

162 studies would focus on generating O26:H11 mutant strains deficient in certain replication or

163 virulence – associated genes, and comparing the competition results from O157:H7 and

164 O26:H11 mixtures, using either O26:H11 wild-type strains or the mutant strains.

165   In conclusion, we have shown that STEC O26:H11 strains were able to effectively outgrow

166 O157:H7 stains in both planktonic and biofilm phases during coexistence via simultaneous

167 inoculation. In the presence of pre-formed O157:H7 biofilms, the O26:H11 strains could still

168 compete with the colonized O157:H7 cells and establish themselves in the mixed biofilms, while

169 serotype O157:H7 appeared to be a poor colonizer if O26:H11 cells pre-occupied the solid

170 surfaces. The fact that the O26:H11 strains have the strong ability to outcompete serotype

171 O157:H7 highlights the potential risk of this non-O157 STEC in food safety and calls for

172 increased attention to the non-O157 STEC biofilms in food processing facilities.

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179   **References**

180   1.   Aidar-Ugrinovich, L., Blanco, J., Blanco, M., Blanco, J.E., Leomil, and L., Dahbi, G.

181   2007. Serotypes, virulence genes, and intimin types of Shiga toxin-producing *Escherichia*

182   *coli* (STEC) and enteropathogenic *E. coli* (EPEC) isolated from calves in Sa˜o Paulo,

183   Brazil. *Int. J. Food Microbiol*. 115(3): 297–306.

184   2.   Allerberger F, Sölder B, Caprioli A, Karch H. 1997. Enterohemorrhagic *Escherichia coli*

185   and hemolytic-uremic syndrome. *Wien Klin Wochenschr*. 109(17):669-77.

186   3.   Bielaszewska M, Zhang W, Mellmann A, Karch H. 2007. Enterohaemorrhagic

187   *Escherichia coli* O26:H11/H-: a human pathogen in emergence. *Berl Munch Tierarztl*

188   *Wochenschr*. 120(7-8):279-87.

189   4.   Brooks, J.T., Sowers, E.G., Wells, J.G., Greene, K.D., Griffin, P.M., Hoekstra, R.M.,

190   Strockbine, N.A., 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in

191   the United States, 1983–2002. *J. Infect. Dis*. 192:1422–1429.

192   5.   Clawson ML, Keen JE, Smith TP, Durso LM, McDaneld TG, Mandrell RE, Davis MA,

193   Bono JL. 2009. Phylogenetic classification of *Escherichia coli* O157:H7 strains of human

194   and bovine origin using a novel set of nucleotide polymorphisms. *Genome Biol*. 10

195   (5):R56.

9

196   6. Habimana O, Heir E, Langsrud S, Asli AW, Møretrø T. 2010. Enhanced surface

197   colonization by *Escherichia coli* O157:H7 in biofilms formed by an *Acinetobacter*

198   *calcoaceticus* isolate from meat-processing environments. *Appl Environ Microbiol.*

199   76(13):4557-9.

200   7. Marouani-Gadri N, Augier G, Carpentier B. 2009. Characterization of bacterial strains

201   isolated from a beef-processing plant following cleaning and disinfection - Influence of

202   isolated strains on biofilm formation by Sakaï and EDL 933 *E. coli* O157:H7. *Int J Food*

203   *Microbiol.* 133(1-2):62-7.

204   8. Mora, A., Blanco, M., Blanco, J.E., Dahbi, G., Lo´pez, C., and Justel, P. 2007. Serotypes,

205   virulence genes and intimin types of Shiga toxin (verocytotoxin)-producing *Escherichia*

206   *coli* isolates from minced beef in Lugo (Spain) from 1995 through 2003. *BMC Microbiol.*

207   7(1): 13–13.

208   9. Rivera-Betancourt M, Keen J. E. 2000. Murine monoclonal antibodies specific for

209   lipopolysaccharide of *Escherichia coli* O26 and O111. *Appl Environ Microbiol.*

210   66(9):4124-7.

211   10. Uhlich G. A, Rogers D. P, Mosier DA. 2010. *Escherichia coli* serotype O157:H7

212   retention on solid surfaces and peroxide resistance is enhanced by dual-strain biofilm

213   formation. *Foodborne Pathog Dis*. 7(8):935-43.

214   11. USDA/FSIS. 2011. Shiga toxin-producing *Escherichia coli* in certain raw beef products.

215   *Fed. Register.* 76(182): 58157-58165.

216   12. Wang, R., Bono, J., Kalchayanand, N., Shackelford, S., and Harhay, D. 2012. Biofilm

217   formation by Shiga – toxin producing *Escherichia coli* O157:H7 and non-O157 strains

218 and their tolerance to sanitizers commonly used in food processing environment. *J. Food.*

219 *Prot.* Accepted on March 26, 2012.

220 13. Werber, D., Fruth, A., Liesegang, A., Littmann, M., Buchholz, U., and Prager, R. 2002. A

221 multistate outbreak of Shiga toxin-producing *Escherichia coli* O26:H11 infections in

222 Germany, detected by molecular subtyping surveillance. *J. Infect. Dis*. 186(3): 419–422.

223 14. Westerman R. B, He Y, Keen J. E, Littledike E. T, Kwang J. 1997. Production and

224 characterization of monoclonal antibodies specific for the lipopolysaccharide of

225 *Escherichia coli* O157. *J Clin Microbiol.* 35(3):679-84.

226 15. Whittam, T. S. 1998. Evolution of *Escherichia coli* O157:H7 and other Shiga toxin-

227 producing *E. coli* strains. p. 195-209. In J. B. Kaper and A. D. O'Brien (ed.), *Escherichia*

228 *coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington,

229 DC.

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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 $\pm$ 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 $10^7 CFU/mL \pm SD$ *(% in the mixture); n=3] and*

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

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

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