Resistance of biofilm- and pellicle-embedded strains of *Escherichia coli* encoding the transmissible locus of stress tolerance (tLST) to oxidative sanitation chemicals

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Abstract

Biofilm formation in food processing plants reduces the efficacy of sanitation. The presence of transmissible locus of stress tolerance (tLST) also enhances resistance of planktonic cells of *Escherichia coli* to sanitation chemicals but the role of tLST in resistance of biofilm-embedded cells remains unclear. This study investigated the link of tLST to biofilm formation and its contribution to resistance of biofilm-embedded *E. coli* to sanitation. Biofilms were formed as single-strain and as dual-strain biofilms in association with *E. coli*, *Aeromonas australensis* or *Carnobacterium maltaromaticum*. Biofilms on stainless steel were compared to floating biofilms formed at the air-liquid interface (pellicles). The resistance of biofilm-embedded tLST positive strains of *E. coli* to chlorine, hydrogen peroxide, and peroxyacetic acid was higher than the resistance of tLST negative strains. Higher biofilm density as measured by crystal violet staining was observed in tLST-positive strains of *E. coli* when compared to tLST negative strains. Biofilm density positively correlated to resistance to disinfectants. The use of confocal laser scanning microscopy detected more compact structure of pellicles compared to solid surface-attached biofilms, resulting in higher chlorine resistance despite the absence of tLST in strains of *E. coli*. Collectively, the findings of this study elucidated the impact of tLST in strains of *E. coli* on biofilm formation and sanitizer resistance. These findings may inform the development of improved sanitization protocols for food facilities.

Keywords: Biofilm formation, transmissible locus of stress tolerance (tLST), locus of heat resistance (LHR), pellicle formation, disinfectants

1. Introduction

Biofilms are surface associated microbial communities where an extracellular matrix of polysaccharides, proteins, lipids, and water provides a three-dimensional structure. The formation of biofilms on surfaces is initiated by attachment, followed by formation of micro-colonies, biofilm maturation, and biofilm dispersion (O'Toole et al., 2000; Watnick and Kolter, 2000). Free-living cells bind to abiotic surfaces as the first stage of biofilm formation (Galié et al., 2018). Second surface structures including type 1 fimbriae, type 3 fimbriae, conjugative pili and curli mediate adhesion (Beloin et al., 2008). Maturation of biofilms is initiated by quorum sensing, which upregulates biosynthesis of the extracellular matrix and the formation of the three-dimensional architecture. Finally, biofilm-embedded cells detach and may colonize other areas (O'Toole et al., 2000). Biofilm formed at air–liquid interfaces are termed floating biofilms or pellicles. In Gram-negative bacteria, pellicle formation has been described for acetic acid bacteria, *Salmonella* spp., *Acinetobacter baumannii*, *Escherichia coli* (Golub and Overton, 2021; Marti et al., 2011; Møretrø et al., 2009; Scher et al., 2005). In *E. coli*, pellicle formation was attributed to the secretion and accumulation of diverse polysaccharides including polymeric β-(1→6)-N-acetyl-D-glucosamine, colanic acid, and cellulose (Beloin et al., 2008).

The biofilm matrix maintains the microbial communities in place even at conditions of strong fluid flow, and thus impedes cleaning. In addition, the biofilm matrix protects biofilm-embedded cells against antimicrobial compounds, and thus impedes sanitation. Biofilms thus contribute to the persistence of bacteria in the food industry and in clinical settings despite regular cleaning and sanitation (Abdallah et al., 2014; Galié et al., 2018; Otter et al., 2015). Biofilm-forming bacteria include pathogens that contribute to foodborne bacterial disease. Strains of *E. coli* O157:H7 isolated from a beef industry during "high event period" (HEP) share the same genotype (Arthur

et al., 2014) and their persistence was linked to biofilm formation (Wang et al., 2014). Similarly, the survival in a meat processing plant of *E. coli* O157:H7 after sanitation increased when the organism was part a biofilm communities (Chitlapilly Dass et al., 2020). Strains of *Salmonella enterica* from beef trim also formed biofilms, which was related to enhanced sanitizer tolerance (Wang et al., 2017).

Research related to biofilm formation by foodborne bacterial pathogens was predominantly conducted with single-strain biofilms, however, biofilm communities generally involve multiple species in food processing facilities. Multi-species biofilms in a meat processing plant were composed of strains of the genera *Brochothrix, Pseudomonas* and *Psychrobacter*, which also commonly occur as meat spoilage organisms (Wagner et al., 2020). *Staphylococcus, Bacillus, Pseudomonas*, among others, also coexisted with *E. coli* O157:H7 in biofilms on the surface of stainless steel and polyvinyl chloride in a meat processing facility (Marouani-Gadri et al., 2009). *L. monocytogenes* also becomes established in multi-species biofilms, e.g. together with *E. coli* in the fish industry and with *Carnobacterium* spp. in meat plants (Rodríguez-López et al., 2015).

The food industry employs multiple measures, particularly hygienic design of equipment and facilities and appropriate cleaning and sanitation protocols with sodium hypochlorite, hydrogen peroxide, or peracetic acid to prevent biofilm formation or to eradicate existing biofilms. Novel or experimental tools include enzymatic disruption of biofilms, physical methods such as hot steam, ultrasound, or surface modification with nanocomposites (Galié et al., 2018; Yuan et al., 2019). Chlorine-based sanitizers are frequently used for sanitation of food processing plants, however, foodborne pathogens including *E. coli* and *Salmonella* Enteritidis develop resistance against chlorine and related oxidizing chemicals when embedded in biofilms (Yang et al., 2016).

Chlorine resistance of *E. coli* and *Salmonella* is also mediated by the transmissible locus of stress tolerance (tLST) (Kamal et al., 2021), previously designated locus of heat resistance (LHR), a ~ 14kb genomic island (Mercer et al., 2015; Wang et al., 2020). tLST-positive and heat resistant strains of *E. coli* were isolated from meat processing plants (Dlusskaya et al., 2011; Guragain et al., 2021) but also from raw milk cheese, clinical setting, and wastewater samples (Peng et al., 2013; Zhi et al., 2016). Some tLST-positive strains of *E. coli* also form strong biofilms (Marti et al., 2017). Taken together, the presence of the tLST in biofilm-embedded cells of *E. coli* potentially further increases sanitation resistance, however, the resistance of biofilm embedded tLST-positive and tLST-negative cells has not been assessed experimentally. It was therefore the aim of the study to investigate whether the presence of tLST in strains of *E. coli* strains increases the tolerance of commonly used disinfectants in mono- or dual-strain biofilms formed on stainless steel and to determine whether pellicle formation by strains of *E. coli* also increases their chorine resistance.

2. Materials and Methods.

2.1. Strains and culture conditions

Strains and their origin are shown in Table 1. Twelve tLST-negative strains of *E. coli*, thirteen tLST-positive strains of *E. coli*, *Aeromonas australiensis* 03-09, *Carnobacterium maltaromaticum* 9-67 and *E. coli* O157:H7 1934 were used in this study. Frozen (-80^{o}C) stock cultures of Gram-negative bacteria were streaked on Luria–Bertani agar plates and incubated in 37°C incubator for 24h, followed by subculture in LB without NaCl (LBNS) broth overnight at 37°C with 200rpm agitation. *C. maltaromaticum* 9-67 was cultivated at 25°C. Selective MacConkey agar plates were used to distinguish non-lactose fermenters (*A. australiensis* 03-09 and *C. maltaromaticum* 9-67) from twenty-five *E. coli* strains. Sorbitol MacConkey plates were used for enumeration of *E. coli* O157:H7 1934.

106 *2.2. Mono- and dual-strain biofilm and pellicle formation*

107 The formation of mono-strain biofilms was observed only for strains of *E. coli*. Because food
108 processing equipment is predominantly constructed with stainless steel (Simões et al., 2010),
109 biofilm formation was observed on food-grade stainless steel coupons. Dual-strain biofilm were
110 formed by mixing one strain of *E. coli* with *A. australiensis* 03-09 or *C. maltaromaticum* 9-67 or
111 *E. coli* O157:H7 1934. Aliquots of each overnight cultures (10µl) were inoculated into 2ml LBNS
112 to achieve the 100-fold diluted bacterial suspension. Stainless steel (SS) coupons (grade 304, No.4
113 finish, 12mm diameter; Stanfos, Edmonton, AB, Canada) were placed into the bottom of a 24-well
114 flat-bottom cell culture plate (Corning, Glendale, Arizona) and, the whole content of bacterial
115 suspension described above was transferred into the plates and incubated at 23.5 $\pm$ 0.3°C for 6d.
116 After 6d, biofilms grown on SS coupons and pellicles formed at the air-liquid interface were
117 harvested with pipette tips and used for cell counts (control), disinfection treatment and biomass
118 quantification. Cell counts were determined after gently washing the SS coupons to remove loosely
119 attached planktonic cells, followed by addition of 2 mL of Dey-Engley (D/E) neutralizing broth to
120 the SS coupons. Biofilm-embedded cells were detached by mixing with 1.64g glass beads and
121 vortexing at maximum speed for 1min. Cell counts were expressed relative to the surface area of
122 the SS coupons of 1.13cm^2. In dual-strain biofilms, differential cell counts were obtained with the
123 selective media indicated above.

124 *2.3. Sanitizers*

125 Three different sanitizers were used in this study, which was diluted from the following stock
126 solutions: 5% (w/v) sodium hypochlorite, 30% (v/v) hydrogen peroxide and 32% (v/v) peracetic
127 acid in acetic acid (Sigma-Aldrich, St. Louis, MO). The final concentration of sanitizers was
128 chosen to achieve a ~ reduction of cell counts by about 1 – 5 log(CFU/mL). Chlorine was diluted

to a final concentration of 800pm and 258ppm in PBS buffer (pH at 6.8) for the treatment of biofilm-embedded and planktonic cells, respectively. The treatment concentration of 2% (v/v) hydrogen peroxide and 0.032% (v/v) peracetic acid were prepared in sterile distilled water. Chlorine test strips (MQuant, Billerica, MA) were used to determine the free chlorine concentration before treatment.

2.4. Curli and cellulose expression

Congo red indicator (CRI) plates were used to evaluate the expression of curli and cellulose production. The preparation of CRI plates was described previously (Wang et al., 2013) composed of 10g/L of Casamino Acids, 1g/L yeast extract, 20g/L Bacto agar, 40mg/L Congo Red and 20mg/L Coomassie brilliant Blue. The cellulose and curli production was determined by streaking overnight cultures on CRI plates and incubating at 23.5 $\pm$ 0.3°C for 6d. The colony morphology of red, brown, pink or white corresponded to both cellulose and curli production, to curli, to cellulose or to neither, respectively (Visvalingam et al., 2017).

2.5. Effect of sodium hypochlorite on planktonic coculture

For the planktonic dual-cultures, overnight cultures of strains of *E. coli* and *A. australiensis* 03-09, *C. maltaromaticum* 9-67 and *E. coli* O157:H7 1934 were equally aliquoted into 5 ml LBNS broth to achieve 100-fold dilution, then the suspension was incubated overnight prior to 25°C for chlorine treatment. The chlorine treatment on planktonic dual cultures with 258ppm sodium hypochlorite were performed as previously described (Visvalingam et al., 2018) with modification. In brief, 100µl mixed-strain overnight cultures were added together with either 100µl sterile water or sodium hypochlorite solution in a 1.5ml microcentrifuge tube. The tube was vortexed at maximum speed for 10s following with 50s incubation at 23.5 $\pm$ 0.3°C. After 1min of treatment, the content of each tube was transferred into a 15ml conical tube containing 1.72ml of sterilized

152 D/E neutralizing broth, followed by vortexing for 15s. Selective agar plates as described above
153 were used after dilution for plating and incubated at 37^{o} C for 18h.

154 For instance, white colonies were detected in Sorbitol MacConkey agar as *E. coli* O157:H7 1934
155 while other *E. coli* strains were presented with dark pink colonies. In MacConkey agar plate, white
156 colonies were observed for *A. australiensis* 03-09 while strains of *E. coli* appeared with dark pink
157 color. Growth of *C. maltaromaticum* 9-67 was inhibited on MacConkey agar plate. Each
158 experiment was repeated three times with independent bacterial cultures (n=3).

159 *2.6. Effect of sodium hypochlorite on mono- and dual-cultures pellicle*

160 Air-liquid interface pellicles were lifted with a pipette tip and loosely attached cells were removed
161 by rinsing three times in LBNS broth. Next, pellicles were treated with 1ml of 800ppm sodium
162 hypochlorite solution or PBS buffer (control) for 1min in 24-well plates. Finally, treated pellicles
163 were lifted and transferred into a 15ml centrifuge tube containing 2ml D/E neutralizing broth and
164 1.64g glass beads. The tube was vortexed vigorously for 1min to disrupt the pellicles. Samples
165 were serially diluted with 0.1% peptone water and spread-plated on selective agar plates before
166 incubation at 37^{o}C for 18h.

167 *2.7. Effect of sodium hypochlorite, hydrogen peroxide and peracetic acid on biofilms formed*
168 *on stainless steel (SS) coupon*

169 Mono- and dual-strain biofilms were formed on SS coupons as described before. At day 6, the
170 coupon was taken out from 24-well plates and rinsed 3 times in LBNS broth to remove loosely
171 attached cells. Then, each coupon was immersed into individual wells containing 1ml of 800ppm
172 chlorine solution for 1min,1ml of 5% hydrogen peroxide solution for 2min, 1mL of 0.032% (v/v)

peracetic acid for 30s or PBS buffer (control) for 1min, respectively. Cell counts after treatments with sanitizing agents were determined as described in section 2.2.

2.8. Quantification of the biomass in biofilms

Biomass was quantified by following the modified crystal violet (CV) method. Briefly, biofilms on SS coupon were washed with sterile water for 3 times to remove loosely associated cells. After that, each coupon was air-dried completely before crystal violet staining. To stain the biofilms, 300µl of 1% (w/v) crystal violet in 95% (v/v) EtOH was added gently and incubated at 23.5 $\pm$ 0.3°C for 20min. Subsequently, stained coupons were rinsed 6 times with sterile water to remove excess stain. One ml 1% (w/v) sodium dodecyl sulfate (SDS) was added and incubated at 23.5 $\pm$ 0.3°C with shaking for 25min to release the dye. The absorbance of each sample was measured at 570nm using a plate reader (Varioskan Flash, Thermo Fisher Scientific). Three independent experiments with technical duplicates were conducted (n=6) for both mono- and dual-strain biofilms.

2.9. Observation of biofilms with confocal laser scanning microscopy (CLSM)

Biofilms formed on SS coupons and pellicles were stained with FilmTracer™ LIVE/DEAD® Biofilm viability kit (Invitrogen Ltd., Paisley, UK), which is employs the cell permeant dye Syto9 and the cell impermeant dye propidium iodide, following the protocol provided by the manufacturer. Stained biofilms and pellicles were imaged by spinning disk confocal microscope, including motorised microscope base (IX-81, Olympus) and confocal scanning unit (CSU 10, Yokagawa). The excitation/emission fluorescence were 482/500nm for SYTO 9 and 490/635nm for PI. Microscopic images of the biofilms were acquired by Perkin Elmer's Volocity software. The mounted samples were observed using a 100X/1.49 oil objective.

2.10. Statistical analysis

Mean values for cell count reduction were collected by three biological replicates. Biomass was quantified by six independent experiments. All analyses were undertaken by two-way analysis of variance (ANOVA) using R statistical package (R Core Team, 2019). Tukey test was used to determine the significant difference with an error probability of 5% ($P<0.05$) as the threshold for significance.

3. Results

3.1. Chlorine resistance of planktonic cells

The tLST protects planktonic cultures of *E. coli* against chlorine (Wang et al., 2020). To determine the chlorine resistance of tLST-positive and tLST-negative biofilm-embedded cells of *E. coli*, an initial screening assessed the ability of 25 strains of *E. coli* to form single-species biofilms on stainless steel (Fig. S1). A majority of strains formed weak biofilms with the absence of visible exopolysaccharides and a cell count of about 10^7cfu/cm^2 or less. Three tLST negative strains, *E. coli* FUA 1866, FUA 1882 and FUA 10043, produced robust pellicles with cell counts of more than 10^8cfu/cm^2 (Fig. S1) and a biofilm structure that was visible without magnification (Fig. 2A). Subsequent experimentation focused on dual strain biofilms formed by strains of *E. coli* with *E. coli* O157:H5 1934, by strains of *E. coli* and the biofilm forming strains of *A. australiensis* 03-09 and *C. maltaromaticum* 9-67, and on pellicles.

To determine whether cultivation of tLST-positive and tLST-negative strains of *E. coli* in mixed culture with *E. coli* O157 1934, *A. australiensis* 03-09 or *C. maltaromaticum* 9-67 impacts chlorine resistance in planktonic cultures, the chlorine resistance of 6 tLST-positive and 6 tLST-negative *E. coli* strains grown in mixed culture with *A. australiensis* 03-09, *C. maltaromaticum* 9-67 or *E.*

coli O157:H7 1934 was tested (Fig. 1). The reduction of viable cell counts of tLST-positive strains of *E. coli* strains ranged from about 1 to 2 $\log_{10}$CFU/ml. In contrast, the lethality of chlorine treatment against of tLST-negative strains of *E. coli* was about 3 to 4log(N_0/N) higher than tLST positive strains (Fig. 1). Therefore, the presence of tLST in *E. coli* strains significantly ($P< 0.05$) increased the resistance of *E. coli* in mixed planktonic cultures to chlorine. The inactivation of *E. coli* strains was not different if *E. coli* was incubated alone or together with *A. australiensis* 03-09, *C. maltaromaticum* 9-67 or *E. coli* O157:H7 1934.

3.2. Pellicle formation, expression of curli, cellulose formation and chlorine resistance

The formation of pellicles at the air-liquid interface was previously observed for *E. coli* (Golub and Overton, 2021). Three of the strains of *E. coli* used in this study produced pellicles after 6d incubation at 23.5 $\pm$ 0.3°C in LBNS (Fig. 2A) and the structure of pellicles was evaluated by confocal laser scanning microscope (Fig. S2B). Pellicle formation was not detected when any of the three pellicle-forming strains of *E. coli* was co-cultured with *A. australiensis* 03-09, but strong pellicle formation was observed when pellicle forming strains of *E. coli* were co-cultured with *E. coli* MG1655 *lacZ*:LHR, *C. maltaromaticum* 9-67 or *E. coli* O157:H7 1934 (Table 2). To investigate whether pellicle embedded cells in mixed-cultures have higher resistance to chlorine compared to pellicle embedded cells in monocultures, we treated monoculture and mixed-culture pellicle embedded cells with 800ppm sodium hypochlorite solution (Fig. 2B). tLST-negative strains of *E. coli* survived in both single-strain and dual-strain pellicles after chlorine treatment with a reduction of viable cell counts of about 2log(N_0/N), which was significantly less than the cell count reduction of tLST negative-strains embedded in biofilms on stainless steel. The resistance of pellicle-embedded strains of *E. coli* to chlorine was not different ($P > 0.05$) if cells

were embedded in pellicles formed by single-strains or mixed-cultures. These data suggest that tLST-negative strains of *E. coli* embedded in pellicles are chlorine resistant.

Cellulose and curli contribute to the pellicle formation by *E. coli* (Golub and Overton, 2021; Hung et al., 2013). Therefore, curli and cellulose expression was assessed on Congo red indicator plates. Pellicle forming strains expressed curli and produced cellulose but not all curli and cellulose positive strains formed pellicles (Table 3). The production of cellulose was more common than expression of curli; with exception of *E. coli* FUA1848 all strains produced cellulose.

3.3. Chlorine resistance of strains of E. coli *in dual-strain biofilms*

The reduction of cell counts of tLST-positive and tLST-negative strains of *E. coli* in dual-strain biofilms after treatment with NaOCl is shown in Figure 3. The chlorine resistance of *E. coli* strains in dual-strain biofilms with *E. coli* O157:H7 1934 was assayed with 800ppm chlorine; biofilms with *A. australiensis* 03-09 and *C. maltaromaticum* 9-67 were treated with 1200ppm chlorine concentration (Fig. 3). The 6 tLST-positive strains of *E. coli* embedded in dual-strain biofilms were more resistant ($P<0.001$) to chlorine when compared to the 6 tLST-negative strains of *E. coli*. In the dual-strain biofilms formed on stainless steel, the biofilm embedded cells of tLST negative strains of *E. coli* were reduced by 3 to 5log CFU/cm^2. In contrast, the reduction of viable cell counts of biofilm-embedded cells of tLST positive strains ranged from 1 to 2.5log CFU/cm^2. The lethality of 800 or 1200ppm chlorine against biofilm embedded dual-strain cultures (Fig. 3) was roughly comparable to the lethality of 258ppm chlorine against dual-strain planktonic cultures with the same strains (Fig. 1).

3.4. Disinfectant resistance of single-strain, dual-strain biofilms and pellicle embedded cells

Peracetic acid and hydrogen peroxide are alternative sanitation agents in the food industry. To assess the resistance of biofilm-embedded cells to these sanitizing agents and to directly compare the resistance of strains of *E. coli* in different biofilm matrices, the resistance of *E. coli* in single-strain or dual-strain biofilms or pellicles was assessed (Fig. 4). In general, tLST-positive strains of *E. coli* were more resistant than tLST-negative strains to all three sanitizing agents irrespective of whether they were embedded in single- or dual strain biofilms (Fig. 4). For tLST-positive strains of *E. coli*, resistance to sanitizers was not strongly impacted by the different biofilm matrices. For tLST-negative strains of *E. coli*, the reduction of cell counts of the same strain in different biofilm matrices differed by up to 2log(CFU/cm^2) but there was no consistent trend as to the biofilm matrix that generated the most resistant cells. The single species biofilm formed by tLST-negative strain of *E. coli* FUA 1838 on stainless steel coupon was significantly more sensitive to sodium hypochlorite and hydrogen peroxide than its dual-strain biofilms ($P < 0.001$) (Fig. 4). The tLST-negative strain *E. coli* FUA10043 embedded in pellicles was equally resistant as tLST-positive strains of *E. coli* embedded in biofilms attached to stainless steel.

3.5. Correlation of biofilm biomass and chlorine resistance

Biomass was quantified with crystal violet staining (Fig. 5). Overall, tLST-positive strains of *E. coli* produced mono- and dual strains biofilms with higher biomass when compared to tLST-negative strains of *E. coli* ($P < 0.01$). Among tLST negative strains, the biomass of single-strain biofilms was less than the biomass of dual-strain biofilms ($P<0.05$) except for the pellicle forming *E. coli* FUA10043. Figure 6 indicates a strong correlation between the biofilm biomass and the chlorine resistance of biofilm-embedded cells of *E. coli* (R=0.903, $P < 0.001$). tLST-positive strains of *E. coli* all clustered at the bottom right of the graph, indicating both a higher biofilm

mass and a higher chlorine resistance in all tLST-positive strains. Therefore, biofilm biomass and chorine resistance are positively associated.

4. Discussion

Experimentation described in this study analysed the biofilm forming ability of *E. coli* in mono- and dual-strain biofilms, and the effect of the presence of the tLST in strains of *E. coli* on resistance to sanitizing agents in biofilm-embedded cells. In planktonic state, proteins encoded by tLST play a protective role on oxidative stress by protein disaggregation and folding (Wang et al., 2020). tLST positive strain of *E. coli* also contain the biofilm-related operons accountable for curli, cellulose and synthesis of polymeric β-(1→6)-N-acetyl-D-glucosamine (Marti et al., 2017) although it remains to be determined whether genes coding for biofilm formation are differentially distributed between tLST-positive and tLST-negative strains. We generally observed a higher biofilm density in tLST positive strains of *E. coli* in comparison to tLST-negative strains, which highly correlates to the enhanced sanitation resistance of biofilm-embedded cells in addition to the tLST-mediated resistance to chlorine and hydrogen peroxide (Fig. 6 and Wang et al., 2020). The association between the presence of tLST and higher biofilm density remains unclear. Multiple tLST variants are currently recognized; several of which also carry *ftsH* (Boll et al., 2017; Kamal et al., 2021; Marti et al., 2017). FtsH contributes to biofilm formation in *P. aeruginosa* (Kamal et al., 2019). Of the strains used in the present study, the tLST in *E. coli* FUA10321 and *E. coli* FUA10323 but not the tLST in *E. coli* AW1.7 include FtsH but formation of biofilms by these strains was roughly equivalent. An alternative explanation for the correlation of biofilm formation ability and the presence of the tLST may relate to the ecological adaptation of these strains. Potentially the selective pressure that maintains the tLST also selects for biofilm formation (Kamal et al., 2021). A high frequency of tLST-positive strains of *E. coli* was isolated from meat and dairy

products after thermal treatment (Boll et al., 2017; Marti et al., 2016; Zhang et al., 2020) as well as from chlorinated wastewater (Zhi et al., 2016). Moreover, oxidative stress agent such as hydrogen peroxide and hypochlorite stimulates biofilm formation in *Acinetobacter oleivorans* and *P. aeruginosa*, respectively (Jang et al., 2016; Strempel et al., 2017). Thus, the role of tLST in the biofilm phenotype that was observed in this study remains subject to future investigations. Irrespective of the mechanisms underlying the increase of biofilm density in tLST positive strains, the presence of tLST enhanced resistance against oxidative stress not only in planktonic cells (Wang et al., 2020) but also biofilm-embedded cells, as indicated by the higher chlorine concentration that was required to reduce viable cell counts. Therefore, biofilm growth of tLST-positive *E. coli* further enhances the chlorine resistance.

The transition of free-living microorganism to a biofilm lifestyle benefits its growth in a hostile condition with limited nutrients. Biofilms form on biotic or abiotic surfaces and also as floating biofilms at the air-liquid interface. The potential mechanisms behind pellicle formation have been explained with regards to buoyancy, the secretion of surface-active agents like surfactants and pellicle attachment to edge of container close to the interface (Armitano et al., 2014). Comparable to biofilms formed on solid surfaces, pellicles are established in several stages. Initially, cells localise at the air-liquid interface by developing floating aggregates. Then cell replication results in the expansion of pellicle at the entire air-liquid interface, followed by EPS secretion and pellicle maturation (Armitano et al., 2014). The formation of pellicles by *E. coli* strains was initially described in uropathogenic *E. coli* (UPEC), enteropathogenic *E. coli* (EPEC) and *E. coli* K-12 (Golub and Overton, 2021; Hung et al., 2013; Wu et al., 2012). Major matrix components of pellicles include curli, cellulose, flagella and type 1 pili (Hung et al., 2013; Weiss-Muszkat et al., 2010). However, the role of pellicles in resistance of *E. coli* to disinfectants has not been studied.

Our study demonstrated that pellicle-embedded tLST negative strains of *E. coli* were equally or more resistant than tLST positive strain of *E. coli* in surface attached biofilms. Whether the formation of pellicles in strains of *E. coli* impacts their virulence remains unknown. However, membrane proteins are overexpressed in another Gram-negative pellicle cells, *A. baumannii*, which potentially influence its virulence and persistence (Marti et al., 2011). Hence, this unique phenotype in *E. coli* strains deserves more investigation.

Biofilm communities in natural environments commonly include multiple species. Interspecific interactions in microbial consortia affect its development, composition, and antimicrobial resistance (Burmølle et al., 2014; Elias and Banin, 2012). In monocultures, *E. coli* O157:H7 1934 did not form biofilms at 15°C after 6d. However, this strain established biofilms when co-cultured with other species at the same incubation condition (Visvalingam et al., 2019). *A. australiensis* 3-09 and *C. maltaromaticum* 9-67, isolated from conveyor belts in a beef facility (Wang et al., 2018), dominated multi-species biofilms (Visvalingam et al., 2019) and showed synergistic effects on biofilm formation when cultivated together with *Salmonella* Typhimurium (Visvalingam et al., 2018). The synergistic interspecific interactions also promote biomass production and thus significantly increase bacterial resistance to disinfectants when compared to single strain biofilms (Burmølle et al., 2006; Van der Veen and Abee, 2011). Biofilms in meat processing plants were reported to include strains of up to 22 genera, indicating that the cleaning and sanitation regime was not efficiently eliminating spoilage and pathogenic bacteria (Fagerlund et al., 2017).

Compared to planktonic state cells, bacterial biofilm cells exhibit higher resistance to sanitizers. One of the most distinctive features that distinguishes biofilm from planktonic cells is the complex structure of the EPS matrix, which represents around 90% of the total biofilm biomass and protects bacterial cells from harsh environment (Pinto et al., 2020). The role of biofilm formation on the

resistance to antimicrobial agents has been extensively studied (Abdallah et al., 2014; Donlan, 2000; Flemming et al., 2016; Kostaki et al., 2012; O'Toole et al., 2000). First, the complex architecture of EPS acts as a physical barrier that limits biocide diffusion to the interior of the biofilm (Bridier et al., 2011). Second, the biofilm matrix reacts with antimicrobial agents, thereby compromising their efficacy (Flemming et al., 2016). Third, the exposure of biofilm-embedded cells to low concentrations of sanitizers supports adaptation and selects for biofilms-embedded cells with enhanced resistance (Bridier et al., 2011; Flemming et al., 2016). Fourth, bacterial cells enclosed in the matrix have a different lifestyle than planktonic cells owing to its low accessibility to nutrients and oxygen (Flemming et al., 2016), which also decreases its sensitivity to biocide reagents (Bridier et al., 2011). The present study demonstrated that the resistance of *E. coli* to sanitation chemicals was highly correlated to biofilm mass or density, which further highlights the role of the biofilm matrix in establishing a diffusion barrier. Moreover, chlorine is inactivated by organic matter (Lambert and Johnston, 2001) and thus likely inactivated before it reaches the interior of the biofilm. The densest biofilm matrices were observed with pellicle-forming and tLST negative strain of *E. coli* (Fig. S2); these strains exhibited resistance to chlorine that was comparable to the resistance of tLST-positive cells embedded in less dense biofilms on stainless steel surface.

Pathogenic microorganisms are a main concern in food industry and their occurrence in biofilm-embedded cells increases their persistence and the risk of food contaminations. In food processing plants, biofilm form on both biotic surfaces and abiotic surfaces like conveyor belts, drying area and floor drain with abundance of moisture and nutrients (Srey et al., 2013), but also on employee gloves, packing materials and animal carcasses (Galié et al., 2018). Microbes that persist in biofilms on food-contact surfaces can consistently contaminate the food products. For example,

biofilms that include Shiga toxin-producing *E. coli* are often found in meat plant associated with equipment surfaces and biofilm dispersal may lead to contamination of beef (Wang et al., 2012). Biofilm formation is also relevant for persistence and dispersal of *L. monocytogenes* onto ready-to-eat meat (Maury et al., 2019). Analysis of an outbreak of listeriosis that was linked to a single meat processing facility documented that isolates that were obtained over a period of 5 year differed in fewer than 11 SNP's (Lachmann et al., 2020), which implies that a single strain persisted in the same facility for 5 years. Hence, the formation of biofilms in food processing increases the risk of foodborne illness.

In conclusion, in this study, we demonstrated that the combination of the presence of tLST and biofilm formation encountered inside single- and dual-strain biofilms profoundly escalate disinfectant resistance. However, the complexity of multi-species biofilms is increased with the additional presence of many other microbial species in a real food processing facility. Thus, further research on biofilms by using a more diverse community of strains and species is necessary, which would benefit the development of methods for controlling bacterial biofilms in food processing ecosystems.

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588

589 **Figure legends**

590 **Figure 1.** Lethality of chlorine treatments to planktonic cells of 12 strains of *E. coli* incubated with
591 *Aeromonas australiensis* 03-09 (black bars), *E. coli* O157:H7 1934 (gray bars) and
592 *Carnobacterium maltaromaticum* 9-67 (white bars). Treatment lethality is expressed as the
593 reduction of cell counts [$\log(N_0/N)$] after treatment with 258 ppm NaOCl. Data are shown as means
594 ± standard deviations for three independent experiments. Values differ significantly ($P<0.05$) if
595 the bars do not share a common superscript.

596 **Figure 2.** Pellicle formation and chlorine resistance of strains of *E. coli.* **Panel A.** Pellicle formed
597 by *E. coli* FUA10043 after 6d at 23.5 $\pm$ 0.3°C in Luria broth. **Panel B.** Reduction of cell counts
598 strains of *E. coli* in pellicles formed by single strain culture (black bars) or in multispecies pellicles
599 formed in Luria broth at 23.5 $\pm$ 0.3°C for 6d, followed by treatment with 800ppm NaClO. Multi-
600 species pellicles were formed by incubation of pellicle forming *E. coli* strains with *E. coli*
601 *lacZ:*LHR (dark gray), *E. coli* O157:H7 1934 (light gray) or *Carnobacterium maltaromaticum* 9-
602 67 (white). Data are shown as means ± standard deviations for three independent experiments.
603 Values differ significantly ($P<0.05$) if the bars do not share a common superscript.

604 **Figure 3.** Reduction of cell counts of tLST-positive and tLST-negative strains of *E. coli* biofilms
605 after treatment with NaClO. *E. coli* strains were incorporated in biofilm produced by *Aeromonas*
606 *australiensis* 03-09 (black bars), *E. coli* O157:H7 1934 (gray bars) and *Carnobacterium*
607 *maltaromaticum* 9-67 (white bars). Biofilms were formed at 23.5 $\pm$ 0.3°C for 6d on stainless steel
608 coupons. Biofilms formed by *Carnobacterium maltaromaticum* 9-67 and *Aeromonas australiensis*
609 03-09 were treated with 1200 ppm of NaOCl, biofilms formed by *E. coli* O157:H7 1934 were
610 treated with 800 ppm NaClO; Data are shown as means ± standard deviations for three independent
611 experiments.

Figure 4. Reduction of cell counts of tLST-positive and tLST negative strains of *E. coli* after chlorine treatment of single species or multi-species biofilms. Bars represent single-species biofilms (black bars) or multi-species biofilms formed with *Aeromonas australiensis* 03-09(dark gray), *E. coli* O157:H7 1934 (light gray) or *C. maltaromaticum* 9-67 (white). Biofilms were formed on stainless steel coupon with Luria broth at 23.5 $\pm$ 0.3°C for 6d; P10043 represents pellicles formed by *E. coli* FUA10043. Biofilms were treated with 800ppm NaClO (**Panel A),** or 5% hydrogen peroxide **(Panel B)** or with 0.032% (v/v) peroxyacetic acid (**Panel C)**. Data are shown as means ± standard deviations for three independent experiments. Values differ significantly (P<0.05) if the bars do not share a common superscript.

Figure 5. Quantification of the biomass of biofilms formed by single species of multi-species biofilms using crystal violet staining. Mono- and dual- species biofilms were formed on stainless steel coupon in Luria broth for 6d at 23.5 $\pm$ 0.3°C. Shown are data for mono-species biofilms (black bars) and multi-species biofilms formed by the strains of *E. coli* indicated and *Aeromonas australiensis* 03-09 (dark gray), *E. coli* O157:H7 1934 (light gray) and *Carnobacterium maltaromaticum* 9-67 (white). Data are shown as means ± standard deviations for three independent experiments. Values differ significantly (P<0.05) if the bars do not share a common superscript.

Figure 6. Correlation of the reduction of cell counts after chlorine treatment and the biofilm biomass. Shown are single species biofilms of strains of *E. coli* (circles) or multi-species biofilms with *E. coli* and *Aeromonas australiensis* 03-09 (triangles), *E. coli* O157:H7 1934 (squares) or *Carnobacterium maltaromaticum* 9-67 (diamonds). The biofilm density of tLST-positive strains is shown with black symbols; the biofilm density of tLST-negative strains is shown with open symbols. Data are shown as means ± standard deviation of three independent experiments. The

line shows the linear regression of all data on the plot; the R-value of the linear regression is also indicated.

Table 1. Strains used in this study.

Group	Strain / origin (reference)
***E. coli* tLST negative**	FUA1838 / wastewater (Wang et al., 2020)
	FUA1848 / wastewater (Wang et al., 2020)
	FUA1860 / wastewater (Wang et al., 2020)
	FUA1866 / wastewater (Wang et al., 2020)
	FUA1869 / wastewater (Wang et al., 2020)
	FUA1882 / wastewater (Wang et al., 2020)
	FUA1888 / wastewater (Wang et al., 2020)
	FUA10038 / wastewater (Wang et al., 2020)
	FUA10043 / wastewater (Wang et al., 2020)
	FUA10046 / wastewater (Wang et al., 2020)
	AW1.7ΔpHR1 / meat processing plant (Pleitner et al., 2012)
	MG1655 (K12) / laboratory reference strain
***E. coli* tLST positive**	FUA10316 / wastewater (Wang et al., 2020)
	FUA10317 / wastewater (Wang et al., 2020)
	FUA10318 / wastewater (Wang et al., 2020)
	FUA10319 / wastewater (Wang et al., 2020)
	FUA10320 / wastewater (Wang et al., 2020)
	FUA10321/ wastewater (Wang et al., 2020)
	FUA10322 / wastewater (Wang et al., 2020)
	FUA10323 / wastewater (Wang et al., 2020)
	FUA10324 / wastewater (Wang et al., 2020)
	FUA10325 / wastewater (Wang et al., 2020)
	AW1.7 / beef processing plant (Dlusskaya et al., 2011)
	AW1.3 / beef processing plant (Dlusskaya et al., 2011)
	MG1655 *lacZ*:LHR / derivative of MG1655 (Wang et al., 2020)
Mixed-species biofilms	*Aeromonas australiensis* 03-09 / beef processing plant (Visvalingam et al., 2019)
	E. coli O157:H7 1934 / beef processing plant (Visvalingam et al., 2019)
	Carnobacterium maltaromaticum 9-67 / beef processing plant (Visvalingam et al., 2019)

Table 2. Formation of floating biofilms (pellicles) by single and mixed cultures of three strains of *E. coli*.

	Strain of *E. coli*		
	FUA 1866	**FUA 1882**	**FUA10043**
Single	+	+	+
E. coli MG1655 *lacZ*:LHR	+	+	+
Aeromonas spp.	-	-	-
E. coli O157:H7	+	+	+
Carnobacterium maltaromaticum	+	+	+

+ pellicle formation; - no formation of pellicles.

Table 3. Curli and/or cellulose expression of bacterial strains tested for biofilm formation. Pellicle forming strains are printed in bold and underlined

Strain of *E. coli*	Curli	Cellulose
FUA1838	+	+
FUA1848	-	-
FUA1860	-	+
FUA1866	+	+
FUA1869	-	+
FUA1882	+	+
FUA1888	-	+
FUA10038	+	+
FUA10043	+	+
FUA10046	-	+
MG1655 *lacZ*:LHR	-	+

Strains of *E. coli* examined for curli and cellulose production are tLST negative

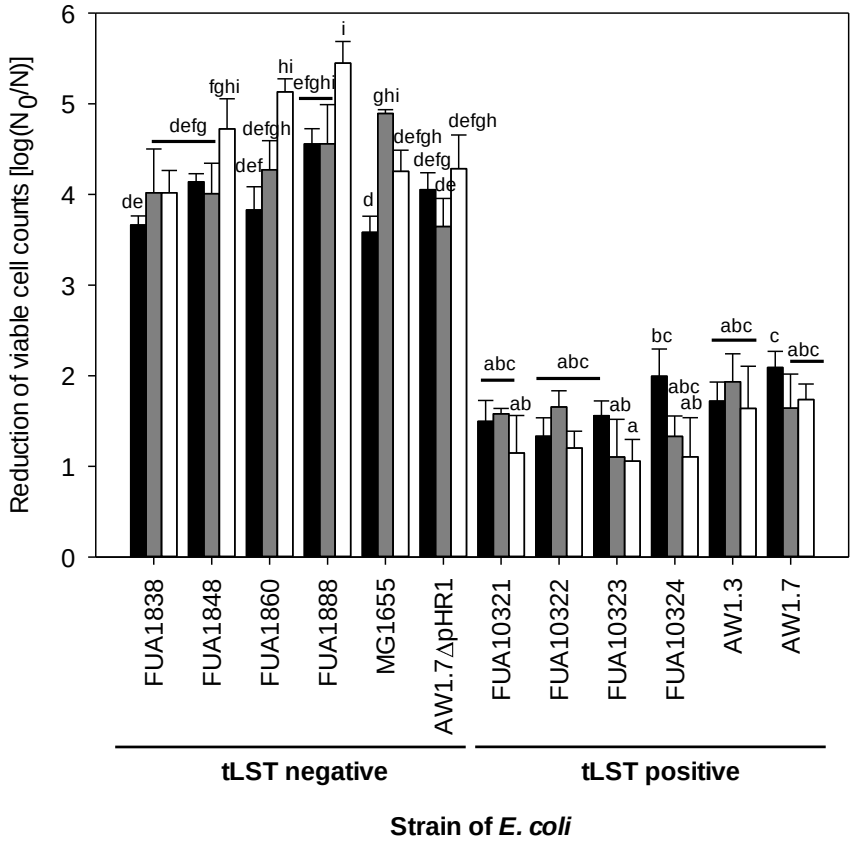
Reduction of viable cell counts [log(N0/N)]
0
1
2
3
4
5
6
de
defg
fghi
def
defgh
hi
efghi
i
d
ghi
defgh
defg
de
defgh
abc
ab
abc
ab
a
bc
abc
ab
abc
c
abc
FUA1838
FUA1848
FUA1860
FUA1888
MG1655
AW1.7ΔpHR1
FUA10321
FUA10322
FUA10323
FUA10324
AW1.3
AW1.7
tLST negative
tLST positive
Strain of E. coli

Figure 1.

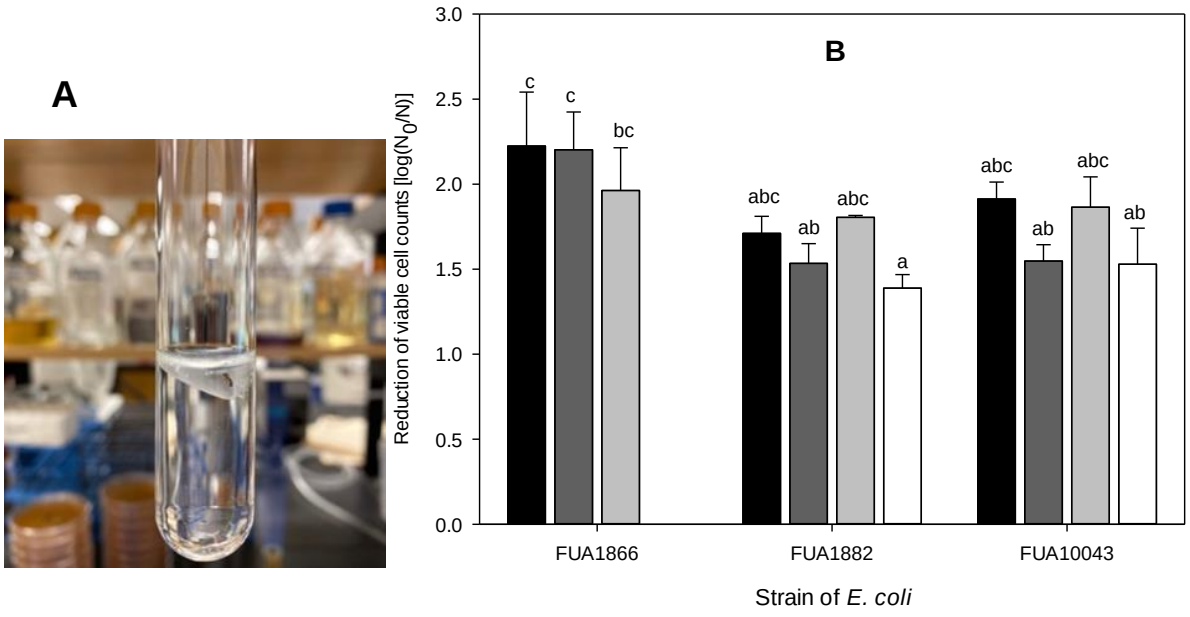
A
B
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Reduction of viable cell counts [log(N0/N)]
c
c
bc
abc
ab
abc
a
abc
ab
abc
ab
FUA1866
FUA1882
FUA10043
Strain of E. coli

Figure 2.

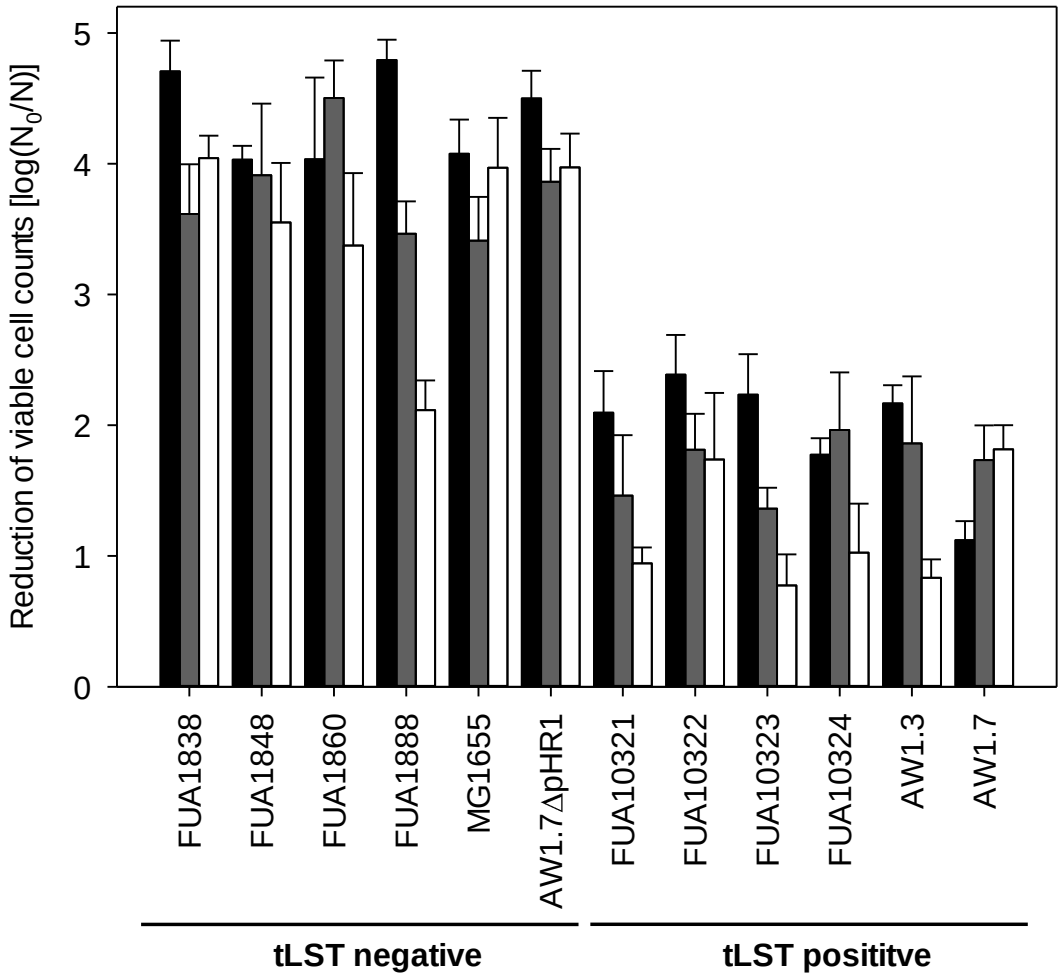
Reduction of viable cell counts [log(N0/N)]
0
1
2
3
4
5
FUA1838
FUA1848
FUA1860
FUA1888
MG1655
AW1.7ΔpHR1
FUA10321
FUA10322
FUA10323
FUA10324
AW1.3
AW1.7
tLST negative
tLST posititve

Figure 3.

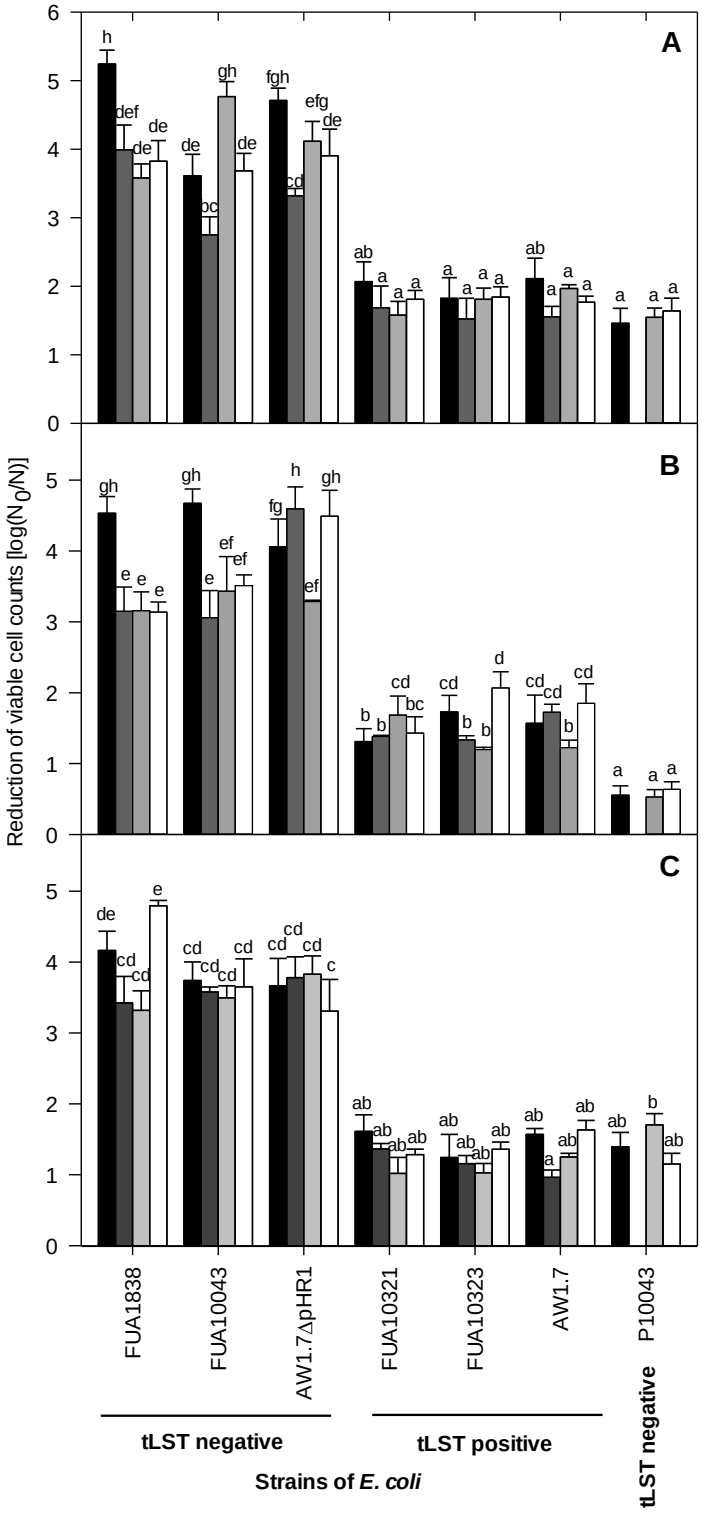
A
B
C
Reduction of viable cell counts [log(N0/N)]
FUA1838
FUA10043
AW1.7ΔpHR1
FUA10321
FUA10323
AW1.7
P10043
tLST negative
tLST positive
tLST negative
Strains of E. coli

Figure 4.

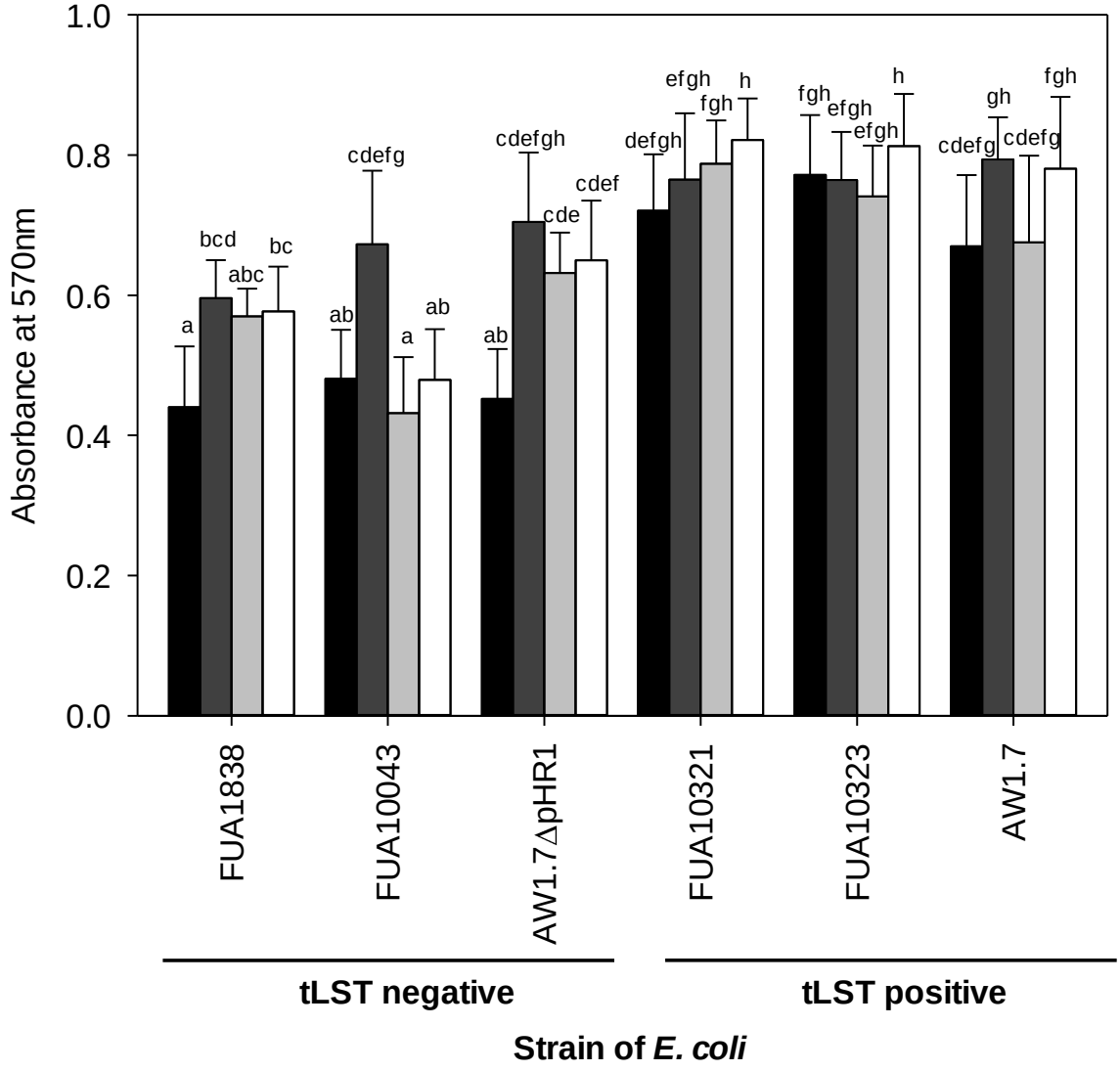
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 570nm
a
bcd
abc
bc
ab
cdefg
a
ab
ab
cdefgh
cde
cdef
defgh
efgh
fgh
h
fgh
efgh
efgh
h
cdefg
gh
cdefg
fgh
FUA1838
FUA10043
AW1.7ΔpHR1
FUA10321
FUA10323
AW1.7
tLST negative
tLST positive
Strain of E. coli

Figure 5.

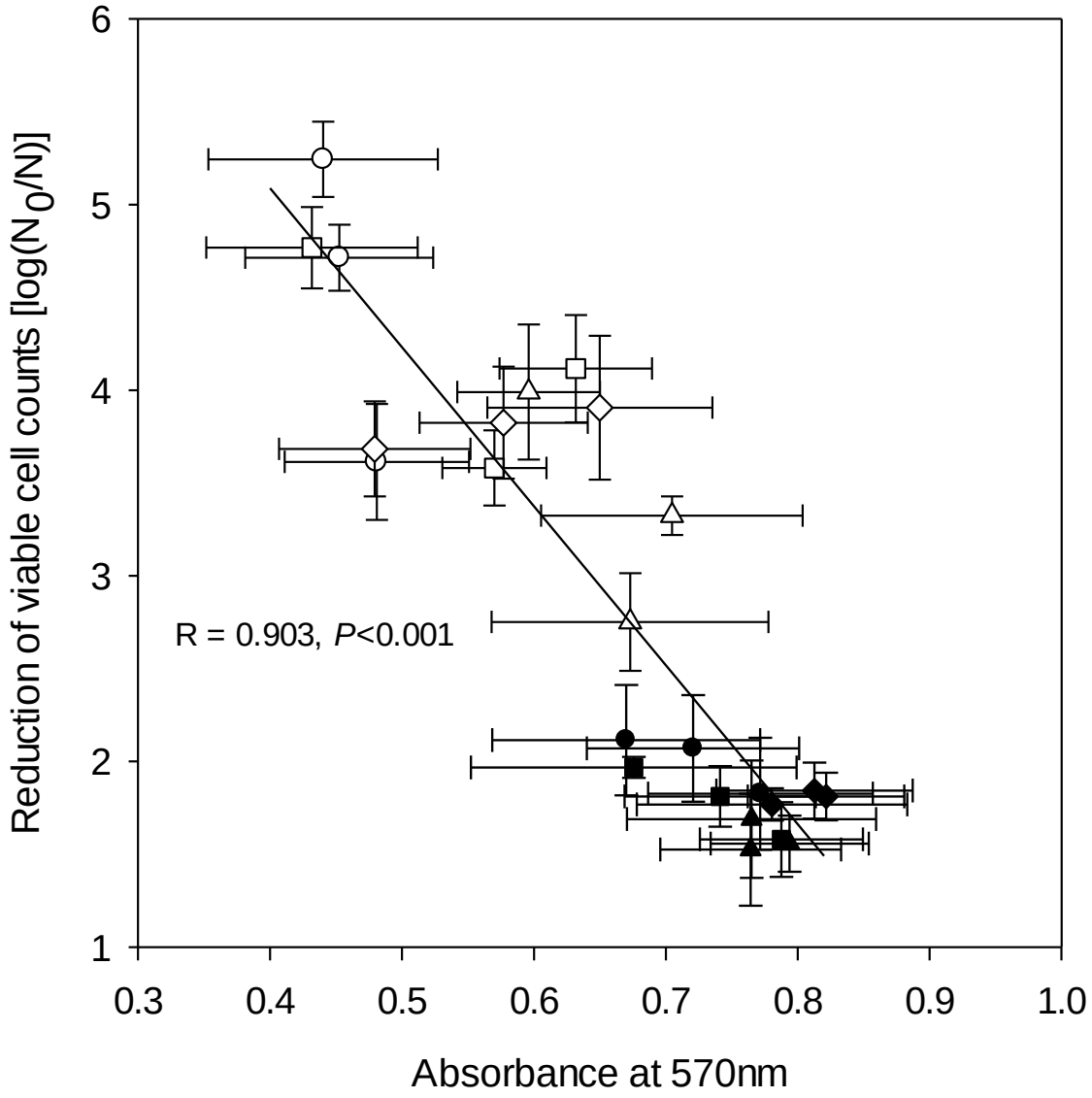
Reduction of viable cell counts [log(N0/N)]
Absorbance at 570nm
R = 0.903, P<0.001
1
2
3
4
5
6
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

Figure 6.