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Impact of long-term quorum sensing inhibition on uropathogenic *Escherichia coli*

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Running title: Quorum sensing inhibitor activity in UPEC

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Synopsis

Background: Quorum sensing is an extracellular bacterial communication system used in the density-dependent regulation of gene expression and development of biofilms. Biofilm-formation has been implicated in the establishment of catheter-associated urinary tract infections (CAUTIs), therefore quorum sensing inhibitors (QSIs) have been suggested as anti-biofilm catheter coating agents. The long-term effects of QSIs in uropathogens is however not clearly understood. We evaluated the effects of repeated exposure to the QSIs cinnamaldehyde, (Z)-4-Bromo-5(bromomethylene)-2(5H)-furanone (C30) and 4-fluoro-5-hydroxypentane-2,3-dione (F-DPD) on antimicrobial susceptibility, biofilm-formation and relative pathogenicity in eight uropathogenic *Escherichia coli* (UPEC) isolates. **Methods:** Minimum inhibitory, bactericidal and biofilm eradication concentrations and antibiotic susceptibility were determined. Biofilm-formation was quantified using crystal-violet. Relative-pathogenicity was assessed in a *Galleria mellonella* model. To correlate changes in phenotype to gene expression, transcriptomic profiles were created through RNA-sequencing and variant analysis of genomes was performed in strain EC958. **Results:** Cinnamaldehyde and furanone-C30 led to increases in susceptibility in planktonic and biofilm-associated UPEC. Relative-pathogenicity increased after cinnamaldehyde exposure (4/8 isolates), decreased after furanone-C30 exposure (6/8 isolates) and varied after F-DPD exposure (one increased and one decreased). 9 out of 21 strains with putative antibiotic cross-resistance were generated. Exposure to cinnamaldehyde or F-DPD reduced expression of genes associated with locomotion, whilst cinnamaldehyde caused an increase in genes encoding fimbrial and afimbrial like adhesins. Furanone C30 caused a reduction in genes involved in cellular biosynthetic processes likely though impaired ribonucleoprotein assembly. **Conclusion:** The multiple phenotypic adaptations induced during QSI exposure in UPEC should be considered when selecting an anti-infective catheter coating agent.

Introduction

Urinary tract infection (UTI) is the most common healthcare associated infection, with between 43% and 56% of cases associated with the presence of an indwelling urethral catheter.[1] The treatment of catheter associated urinary tract infection (CAUTI) is complicated by the emergence of uropathogenic *E. coli* (UPEC) exhibiting multiple antibiotic resistances.[2] In Europe and the US 50,000 people a year lose their lives due to antibiotic-resistant pathogens, with that number rising to 700,000 worldwide.[3] This number is predicted to reach 10 million deaths by 2050 if alternative therapies are not found.[3] There has therefore been substantial interest in the development of strategies to help prevent CAUTI that avoid the use of antibiotics.

Biocide-impregnated catheter coatings have shown promise in reducing the incidence of CAUTI in clinical trials.[4] However, concerns over the selection of biocide-resistant bacterial populations, in addition to the induction of cross-resistance to third party agents such as antibiotics, has led to the search for new strategies.[5] An alternative approach is to use quorum sensing inhibitors (QSIs) as anti-infective catheter coatings. Quorum sensing (QS) is a process by which bacteria produce and detect extracellular signalling molecules and coordinate their behaviour in a cell density dependent manner.[6] QS has been shown to be an important contributor to the formation of bacterial biofilms and expression of virulence factors in certain bacterial species.[7] QSIs disrupt such communicative processes and provide a potential strategy to prevent the establishment of biofilm associated infections such as CAUTI, whilst exhibiting limited cytotoxic effects since they are generally specific to their bacterial targets.

Quorum sensing in UPEC is mediated by autoinducer-2 (AI-2)-based signaling[8] AI-2 consists of derivatives of 4,5-dihydroxy-2,3-pentanedione (DPD)[9] with the synthase enzyme LuxS present in more than 500 bacterial species.[8-9] AI-2 production is directly correlated to biofilm production in *E.coli* through regulation of the motility quorum-sensing regulator gene *mqsR*

which encodes an mRNA interferase.[10] DPD analogues have been shown to prevent AI-2 binding to the periplasmic receptor LsrB[11] disrupting AI-2 based transduction and have exhibited anti-biofilm activity in *E. coli.*[12] We have demonstrated previously that 4-fluoro-5-hydroxypentane-2,3-dione (F-DPD), a novel fluoro DPD analogue, disrupts AI-2-based QS.[13, 14] A small number of diverse natural compounds have also been shown to inhibit AI-2-based signalling. (Z)-4-Bromo-5(bromomethylene)-2(5H)-furanone-C30 (furanone-C30), produced by the red alga *Delisea pulchra*, reduced AI-2 based QS in *E. coli* as well as decreasing swarming motility and biofilm-formation.[15] Cinnamaldehyde, which is obtained from the bark of the cinnamon tree, has also been shown to interfere with AI-2 based signaling[16] and is believed to modulate virulence factor expression in UPEC decreasing urothelial cell attachment and invasion.[17]

Exposure of bacteria to sub-lethal concentrations of antimicrobials, such as found when compounds leach from a coated medical device surface forming a gradient, creates a selective pressure which can select for intrinsically resistant mutants or induce phenotypic adaptations in bacteria leading to decreased susceptibility. The risks associated with sub-lethal concentration exposure of bacteria to biocides has been extensively studied.[18, 19]Anti-virulence strategies such as QS inhibition are attractive therapeutic alternatives to antibiotics and biocides due to a perceived mitigation of this selective pressure. However many of these compounds also display bacteriostatic and bactericidal capabilities[20, 21] and the effects of long-term exposure to QSIs has not been evaluated in bacterial pathogens.

The current study therefore aimed to quantify the effects of QSI exposure in eight UPEC isolates. Specifically we will determine impact on antimicrobial and anti-biofilm susceptibility, the induction of antibiotic cross-resistance in addition to changes in biofilm-formation and relative-pathogenicity. With the aim of correlating phenotypic changes with omics data the

transcriptome and genome of isolate EC958, a virulent multi-drug resistant strain of UPEC was compared before and after QSI exposure.

Methods

Bacteria and chemicals. Six UPEC clinical isolates (EC1, EC2, EC11, EC26, EC28 and EC34) previously isolated from urine during UTI, belonging to the O25:H4-ST131 clone (Stepping Hill Hospital, Stockport, UK) and two laboratory characterised UPEC isolates (EC958 and CFT073) were used in the investigation.[22-24] Bacteria were cultured on Muller-Hinton agar (MHA; Oxoid, UK) or in Muller-Hinton broth (MHB; Oxoid, UK) and incubated aerobically at 37 °C for 18 h at 100 rpm. Cinnamaldehyde was purchased from Sigma–Aldrich (Poole, UK). (Z)-4-Bromo-5(bromomethylene)-2(5H)-furanone-C30 (furanone-C30) was synthesised as described previously.[25] 4-fluoro-5-hydroxypentane-2,3-dione (F-DPD) was synthesised as described previously.[13] Furanone-C30, cinnamaldehyde and F-DPD were dissolved at 1 mg/mL in water and filter sterilised prior to use.

Long-term exposure of bacteria to quorum sensing inhibitors. Bacteria were repeatedly exposed to QSIs using a standardised antimicrobial gradient plating system[19,24, 26-29] adapted from that of McBain *et al.*[26] In brief, 100 µL of an MBC concentration solution of QSI was added to an 8- by 8-mm well in the centre of a 90-mm agar plate. Bacterial pure cultures were radially inoculated in triplicate from the edge of the plate to the centre prior to static incubation for 2 days aerobically at 37°C. The biomass from the inner edge of the annulus of bacterial growth, representative of the highest QSI concentration at which growth could occur, was removed and used to inoculate a new QSI-containing plate, as outlined above. This process was repeated for 12 passages (P12). Control isolates passaged 12 times on QSI-free medium were also included (C12). Passaging was performed using three biological replicates

for each isolate. Bacteria were archived in 50 % glycerol at -80 °C before and after passage for subsequent testing.

Minimum inhibitory and minimum bactericidal concentrations. MIC and MBC were determined as described previously.[27] The MIC was defined as the lowest concentration of QSI for which growth was completely inhibited, determined as a lack of turbidity relative to a sterile negative control. To determine MBC, aliquots (5 µL) were taken from the wells of the MIC plate and were spot plated in triplicate onto Muller Hinton Agar (MHA). The plates were incubated statically for 18 h at 37ºC. The lowest test concentration for which visible bacterial growth was completely inhibited was deemed the MBC. Three biological replicates and two technical replicates were performed for each isolate.

Minimum biofilm eradication concentration. Minimum biofilm eradication concentrations were determined using the Calgary biofilm device (CBD) as described previously.[28] MBEC was defined as the lowest concentration of QSI for which re-growth was completely inhibited, viewed as turbidity relative to a sterile negative control. Three biological replicates and two technical replicates were performed for each isolate.

Catheter biofilm model. Method was adapted from Nweze *et al,*[29] catheter sections were cut into segments (1 cm) halved longitudinally and autoclaved. Overnight cultures of bacteria were made in 20 mL MHB, pelleted at 13,000 x g and washed twice in 5 mL PBS and resuspended in 5 mL PBS at OD_{600} 0.18 (10^7 cells/ml). In a 12 well plate, catheter sections were pre-coated with 4 mL foetal bovine serum (FBS) for 24 h at 37 ºC and 30 rpm. FBS was aspirated and 4 mL bacterial suspension was added before incubation for 90 min at 37 ºC (adhesion phase). Sections were removed and placed in a 12 well plate containing 4 mL MHB. Plates were incubated for 48 h at 37 ºC and 30 rpm (biofilm-formation phase) prior to staining

with 3 mL of crystal violet solution at room temperature for 30 minutes. The crystal violet solution was aspirated and sections were gently washed 3 times by submerging in 4 mL of PBS, allowed to air dry for 1 h at room temperature prior to solubilising in 4 mL of 100 % ethanol and determining $A_{600.}$ Two biological replicates and four technical replicates were performed for each isolate. Biofilm formation for the unexposed P0 isolate and the P12 QSI exposed isolates was statistically compared to the control passaged isolate C12 using an ANOVA with post hoc Tukey analysis $p \leq 0.05$.

***Galleria mellonella* pathogenicity assay.** The pathogenesis model was performed as described in Peleg *et al.*[30] Final larval-stage *G. mellonella* were obtained from Live Foods Direct, Sheffield, UK. Treated larvae were incubated in a petri dish at 37 °C and the number of surviving individuals was recorded daily. An untreated group and a group injected with sterile PBS were used as controls. The experiment was terminated when at least two individuals in a control group had died or after 7 days of incubation. Two biological replicates were used per isolate to inoculate 12 caterpillars each and significance in death rate was calculated using a log-rank reduction test ($p \leq 0.05$).

Antibiotic susceptibility. Bacterial susceptibility was determined for trimethoprim sulfamethoxazole (25 µg), nitrofurantoin (50 µg), ciprofloxacin (10 µg), and gentamicin (200 µg). Antibiotic susceptibility tests were performed according to the standardized BSAC disc diffusion method for antimicrobial susceptibility testing.[31] Three biological replicates with two technical repeats were performed for each isolate.

RNA sequencing. RNA was extracted from 30 mL of a mid-log phase (OD_{600} 0.3) culture of *E. coli* grown in MHB at 37°C and 100 rpm. Three biological replicates were performed for each isolate. RNA was extracted using the TRIzol plus RNA purification kit (Thermofisher, UK). Library preparations, sequencing reactions, and bioinformatic analysis were conducted at GENEWIZ, LLC. (NJ, USA). rRNA depletion was performed using

Ribozero rRNA Removal Kit. RNA sequencing library preparation used the NEBNext Ultra RNA Library Prep Kit. Sequencing libraries were validated using the Agilent Tapestation 4200 (Agilent Technologies, USA), and quantified by using Qubit 2.0 Fluorometer (Invitrogen, CA). Samples were sequenced using a HiSeq 2x150 Paired End (PE) configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS). Data were de-multiplexed using Illumina's bcl2fastq 2.17 software. Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. Reads were mapped to the reference genome available using the STAR aligner v.2.5.2b. Unique gene hit counts were calculated by using feature Counts from the Subread package v.1.5.2. DESeq2 gave a comparison of gene expression between the sample group. The Wald test was used to generate p-values and Log2 fold-changes. Genes with adjusted p-values < 0.05 and absolute log2 fold-changes > 1 were called as differentially expressed genes for each comparison. Statistical overrepresentation tests were performed using the Fishers exact test to determine significant changes in biological function from sets of differentially expressed genes. This was performed using PANTHER.[32]

Genome sequencing

Genome sequencing was performed by MicrobesNG Birmingham, UK. DNA was extracted using a DNeasy kit according to manufacturers instructions (Qiagen, UK). DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified

using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on an Illumina instrument using a 250bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15. [33] De novo assembly was performed on samples using SPAdes version 3.7, [34] and contigs were annotated using Prokka 1.11[35]

Results

QSI susceptibility of UPEC in planktonic and biofilm states. MIC, MBC and MBECs were determined for all test isolates before (P0) and after repeated QSI exposure (P12) and were compared to the respective control passaged on QSI free media (C12; Tables 1-3). Control passaged isolate (C12) was also compared to unexposed parent isolate (P0) to ascertain the effects of the passaging procedure alone.

After repeated QSI exposure there was a ≥2 fold-decrease in MIC in 8/8 isolates for cinnamaldehyde or furanone-C30 indicating an increase in susceptibility compared to their respective passaged controls (C12) (Table 1). There were no significant changes in MIC induced by F-DPD exposure. For MBC (Table 2) QSI exposure led to a ≥2 fold-decrease in 8/8 isolates for cinnamaldehyde and 6/8 isolates for furanone-C30 when compared to the respective passaged control C12. In contrast 1/8 isolates showed a ≥2 fold-increase in MBC after exposure to F-DPD indicating reduced susceptibility. In terms of MBEC (Table 3) there was a ≥2 fold-decrease in 8/8 isolates for cinnamaldehyde and 6/8 isolates for furanone-C30 compared to the passaged control (C12). There were no significant changes in MBEC induced by F-DPD exposure.

A ≥2-fold decrease in susceptibility was observed at MIC in control passaged isolates (C12) compared to the unexposed parent strains (P0) in 8/8 isolates for both cinnamaldehyde and furanone C30 and in 6/8 for F-DPD. There was a ≥2-fold decrease in susceptibility at MBC in

7/8 isolates for cinnamaldehyde, 8/8 for furanone C30 and 6/8 for F-DPD. At MBEC we observed a ≥2 fold decrease in susceptibility 8/8 cinnamaldehyde, 3/8 furanone C30 and saw 2/8 increases in susceptibility for F-DPD.

The impact of QSI exposure on UPEC biofilm-formation. Biofilm-formation was determined via a crystal violet biofilm assay for each UPEC isolate before and after repeated QSI exposure (Figure 1). Unexposed isolates displayed varying biofilm forming capabilities prior to exposure with EC1 showing the highest level of biofilm-formation followed by EC26 > EC11 > CFT073 > EC958 > EC2 > EC34 and EC28. QSI exposure led to significant ($p \leq 0.05$) decreases in biofilm-formation on the catheter surface for 1/8 isolates for cinnamaldehyde (EC34), 3/8 isolates for furanone-C30 (EC28, EC34, EC958) and 3/8 isolates for F-DPD (EC11, EC34 and EC958) when they were compared to their respective passaged controls (C12). When comparing the unexposed parent strain (P0) to the control passaged isolate (C12) an increase in biofilm formation was observed in 5/8 isolates after passaging.

Relative-pathogenicity of UPEC after long-term QSI exposure. A *G. mellonella* waxworm model was used to determine relative-pathogenicity in UPEC isolates (Figure 2). Data indicate that prior to QSI exposure, EC2 was the least pathogenic and EC1 and EC958 were the most pathogenic isolates. Cinnamaldehyde exposure induced significantly (log-rank $p \leq 0.05$) increased relative-pathogenicity in 4/8 isolates (EC2, EC11, EC26, EC28) when compared to the respective control isolate C12. F-DPD exposure induced significantly increased pathogenicity in 2/8 isolates (EC11, EC26) and significantly decreased pathogenicity in 2/8 isolates (EC34, CFT073). Furanone-C30 was the only QSI to induce only significant decreases in pathogenicity which occurred in 6/8 isolates (EC1, EC11, EC26, EC34, EC958, and CFT073). No significant difference in relative pathogenicity was observed when comparing the unexposed parent strain (P0) to the passaged control strain (C12).

Changes in antibiotic susceptibility after QSI exposure. Isolates were classed as resistant, intermediate, or sensitive to each antibiotic as defined by BSAC breakpoints.[31] Antibiotic susceptibility was determined for UPEC isolates before and after exposure to each QSI (Table 4). Data indicate that cinnamaldehyde exposure induced EC26 to become resistant to gentamicin and EC28 to become intermediately resistant to gentamicin. Exposure to furanone-C30 induced gentamicin resistance in 2/8 isolates (EC26 and EC34) and intermediate resistance in 1 isolate (EC28). F-DPD exposure induced gentamicin resistance in 2/8 isolates (EC26 and EC28). Cinnamaldehyde exposure induced CFT073 to become intermediately resistant to trimethoprim sulfamethoxazole as did furanone-C30 exposure. There were cases where isolates that were initially resistant to trimethoprim sulfamethoxazole became more susceptible after QSI exposure. This occurred in EC2 after exposure to furanone-C30 and F-DPD. This was also observed in EC28 for ciprofloxacin after cinnamaldehyde and F-DPD exposure. There was no observed change in BSAC classification of susceptibility in the control C12 isolate when compared to the unpassaged parent strain P0.

Alterations in EC958 genome and transcriptome after QSI exposure.

Variant analysis of the EC958 genome was performed after long-term QSI exposure (P12) or repeated passage in a QSI free environment (C12) relative to an unexposed parent strain (P0, Table 5). Exposure to cinnamaldehyde selected for a mutation in RNA chaperone *proQ* and dnaX encoding the tau subunit of DNA polymerase III. Mutations in a DUF2931 family protein of unknown function were acquired after exposure to all QSIs in addition to the control passage isolate. Silent mutations in *entS,* part of the EntS/YbdA MFS enterobactin transporter system were observed after cinnamaldehyde or furanone exposure and in the control passaged isolate**.**

Transcriptomic data comparing the QSI exposed bacteria (P12) to the passage control (C12), allowing us to determine the effect of QSI exposure whilst controlling for passaging, showed the following; during cinnamaldehyde exposure 368 genes were significantly ($p \leq 0.05$) upregulated and 329 genes were significantly downregulated. GO enrichment analysis of biological function (Table 6) showed a significant downregulation in locomotion (25 genes), chemotaxis (15 genes), bacterial flagellum associated motility (14 genes) and reactive nitrogen species metabolic processes (16). An increase in the expression of genes associated with toxin biosynthetic processes (7 genes), drug catabolic processes (24 genes) and responses to stress (91 genes, Table 6) was observed after cinnamaldehyde exposure. Notably there was a substantial decrease in genes associated with flagellar synthesis and function (*fliCDFGKMNS, flgCGK* and *motA*), chemotaxis (*cheABRWYZ*), cell wall synthesis associated proteins (*mltB, flgJ* and *lysM)*, iron sulphur cluster synthesis (*sufC*) and multidrug efflux transporter systems (*acrAB* and *mdtEF* Table S1). We observed an increase in the expression of genes encoding fimbrial-like adhesin and associated outer membrane usher proteins *yehB* and *yehD*, a *fimA* homologue, and in afimbrial adhesin associated proteins *afaD* and *afaC* (Table S1).

Similarly, exposure to F-DPD caused a significant downregulation in locomotion-related genes with 25 genes differentially expressed including those associated with the flagellum assembly and motility (*fliCDFGHKMNS, flgCHJ* and *motA*) and chemotaxis (*cheABRWYZ*; Table 6 and Table S1). Multidrug efflux transporter system *mdtEF* was downregulated and afimbrial adhesin associated proteins *afaC afaD* and *afaE* were upregulated (Table S1). Exposure to Furanone-C30 led to a significant reduction in a large number of genes associated with translation (34 genes) including ribosomal small (9 genes) and large (13 genes) subunit assembly (Table 6, Table S1). There was an increase in gene expression associated with IMP biosynthetic processes (7 genes), reactive nitrogen species metabolic processes (7 genes), drug catabolic processes (10 genes) and carbohydrate catabolic processes (14 genes, Table 6).

Separately, to determine the effect of passaging, when comparing the control passaged isolate C12 to the unexposed parent strain (P0) increases in expression of genes associated with ribosome assembly (19 genes), cellular biosynthetic processes (156 genes) and cellular respiration (33 genes) were observed as was a decreases in expression of genes associated with reactive nitrogen species metabolic processes (10 genes) (Table 6, Table S1).

Discussion

QSI exposure induces changes in susceptibility in planktonic UPEC. Exposure of UPEC to a panel of QSI's was performed using a gradient plating system.[24] Cinnamaldehyde induced a high frequency of >2-fold increases in susceptibility at MIC, MBC, and MBEC (Tables 1 and 2). Cinnamaldehyde has been previously shown to cause oxidative damage to and alter the fatty acid composition of the *E. coli* cell membrane.[36-37] This may have compromised the structural integrity of the membrane and increased cell permeability, providing a possible mechanism for the observed increase in susceptibility. RNA sequencing of the cinnamaldehyde exposed EC958 strain revealed a downregulation of *sufC* which is involved in iron-sulphur cluster synthesis[38] (Table S1). Decreased SufC activity has been shown to cause increased susceptibility to ROS and DNA damage in certain bacteria.[38] As cinnamaldehyde's mechanism of action depends on oxidative damage[37] a decrease in SufC production may explain the increase in cinnamaldehyde susceptibility. Furthermore, there was a reduction in cellular efflux systems *acrAB* and *mdtEF* and cell wall synthesis associated proteins *mltB, flgJ* and *lysM* which may culminate in increased cell wall permeability and a reduction in efflux-mediated defence (Table 6, Table S1).

There were increases in susceptibility for all of the UPEC isolates when exposed to furanone-C30 at MIC level and 6 increases observed at MBC (Table 1-2). There is minimal previous report of the response of *E. coli* to furanone-C30 outside its immediate inhibitory influence on

QS and biofilm-formation.[39] The furanone exposed EC958 isolate was observed to have significantly decreased expression of *mltA*, a murein transglycosylase, which plays a role in enlargement of the murein sacculus[40] (Table S1). Impairment of sacculus formation could lead to a loss in cell integrity and increased cell permeability leading to increased antimicrobial susceptibility. The control passaged isolate (C12) showed decreased antimicrobial susceptibility when compared to the unexposed parent strain (P0) this may be due to an observed reduction in porin expression (*ompF* and *ompC*) impairing diffusion of QSIs across the membrane and subsequent interaction with intracellular targets (Table S1).

Biofilm-formation and susceptibility in UPEC after QSI exposure. QS in *E. coli* is mediated by AI-2 and it has been shown that AI-2 production is directly correlated to biofilm-formation and motility.[10] Cinnamaldehyde is considered to interfere in AI-2 based signaling and can reportedly further disrupt biofilm-formation by reduced accumulation of EPS.[41] There was an increase in biofilm susceptibility for all isolates after cinnamaldehyde exposure in the current investigation (Table 3) which correlated with a decrease in biofilm-formation for 1/8 isolates on the catheter surface (Figure 1). In a previous study cinnamaldehyde exposure caused an increase in biofilm cell signaling protein *bhsA* and type-1 fimbriae after 4 h.[42] In our study, we found an upregulation of fimbrial like adhesin *yehB*, a *fimA* homologue, and upregulation of fimbrial outer membrane usher protein *yehD* (Table S1). We also observed an increase in afimbrial adhesin associated proteins *afaD* and *afaC.* There was a decrease in numerous genes associated with flagella synthesis, motility and chemotaxis in addition to transcriptional activator *csgD,* which controls cellulose and curli biosynthesis[43] (Table 6, Table S1). Variant analysis of the EC950 genome after cinnamaldehyde exposure revealed a mutation in RNA-binding protein ProQ which plays a role in small RNA-mediated control of gene expression in bacteria (Table 5). ProQ mutation has been shown to decrease expression of a number of flagella associated proteins in extracellular *Salmonella enterica* serovar Typhimurium although

341 the role in *E. coli* is not fully understood.[44] A reduction in motility and impaired cellulose
342 synthesis may reduce biofilm initiation, EPS production and maturation. This would explain
343 the decreases in biofilm-formation and increases in biofilm susceptibility observed in the
344 cinnamaldehyde adapted UPEC isolates.

345 After furanone-C30 exposure small magnitude increases in biofilm susceptibility were
346 observed in 6/8 isolates (Table 3) whilst biofilm-formation decreased for 3/8 isolates (Figure
347 1). *E. coli* biofilm-formation has been shown previously to be inhibited by furanone-C30[15, 45]
348 partially due to a down regulation in genes associated with chemotaxis, flagellar synthesis, and
349 motility. We did not, however, find significant downregulation of genes associated with these
350 processes in furanone adapted EC958 but did see downregulation in a number of genes
351 associated with translation initiation and cellular biosynthetic processes which may directly
352 impact growth rate (Table 6).

353 F-DPD is an analogue of AI-2 and inhibits QS by competitively blocking the AI-2 receptor.[13]
354 In the current study, exposure to F-DPD did not induce any change in biofilm susceptibility
355 however we observed a reduction in biofilm-formation in 3/8 isolates on the catheter surface
356 (Table 3, Figure 1). After F-DPD adaptation there was a reduction in flagellar synthesis and
357 motility associated genes including *fliCDFGHKMNS*, *flgCHJ* and *motA* (Table 6, Table S1).
358 This reduction in motility would impair biofilm initiation and may result in the decrease in
359 biofilm-formation observed in the F-DPD adapted isolates.

360 The observed increase in biofilm formation observed when comparing control passaged strain
361 (C12) to the unexposed parent isolate (P0) is likely due to the observed increase in cellular
362 respiration enhancing growth, cellular biosynthesis and therefore replicative potential during
363 biofilm establishment (Table 6, Figure 1). Increased biofilm formation may consequently lead

to a reduction in biofilm susceptibility, which may be further exacerbated by reduced porin expression on a cellular level (Table 3, Table 6, Table S1).

Changes in antibiotic susceptibility after QSI exposure in UPEC. Decreased susceptibility to antibiotics resulting in a change in BSAC classification occurred in 9 out of a possible 63 cases (Table 4). Increased antibiotic susceptibility where resistance was reversed to a susceptible or intermediate classification occurred in 4 out of a possible 33 cases (Table 4). Previous studies have shown that exposure to cinnamaldehyde upregulated the expression of several antibiotic resistance genes including *marRAB* and *mdtEF* in *E. coli*.[42] We also observed overexpression of *marA* in the cinnamaldehyde exposed EC958 isolate in addition to upregulation of *mdtN* (Table S1) which has been associated with multidrug resistance previously however isolate EC958 did not show induced antibiotic resistance after QSI exposure.[42] Furanone-C30 exposure resulted in an overall reduction in biosynthetic processes within the bacterial cell which may contribute towards some of the antibiotic insusceptibility observed in our UPEC isolates. Treatment with F-DPD has been previously shown to increase rifampicin resistance in *E. coli* due to an increase in mutation rate plasticity via increased modulation of mutational hotspots by Dam methylase[14] which may confer a generalised mechanism of resistance towards different antibiotics. In the current investigation, there were 4 cases where QSI exposure led to increase in the susceptibility of isolates (EC2 and EC28) to certain antibiotics (trimethoprim and ciprofloxacin). Antibiotic cross-protection after antimicrobial exposure has been demonstrated previously[27-28], and is theorised to be caused due to a generalised increased cellular permeability. Variant analysis of EC958 genomes indicated that we did not select for any mutations that have been previously associated with mechanisms of resistance towards the tested antibiotics.

Altered relative-pathogenicity in QSI adapted UPEC. Virulence factors that influence biosynthesis, motility, surface adhesion and toxin production have been shown to be regulated

by QS in *E. coli*.[46] Long-term exposure to cinnamaldehyde induced increased pathogenicity in 4 UPEC isolates which may be due to an increase in adhesin associated proteins *yehB*, *yehD*, *afaD* and *afaC* facilitating cell entry (Table S1). Furanone-C30 was the only QSI to induce only decreases in pathogenicity which occurred in 6/8 isolates (Figure 2). Furanone-C30 has been shown to repress major virulence factors such as proteasases *lasA* and *lasB*, chitinase (*chiC*), and pyocyanine synthesis *phzABCDEFG* in *P. aeruginosa*,[47] but there is no previously reported impact on virulence factor production in UPEC. RNA sequencing of EC958 revealed a reduction in a number of translation associated proteins and a resulting decrease in biosynthetic processes within the cell which may impair replication and thus survival within the waxworm model, however we did not identify change in regulation of any specific virulence factors from our DE gene expression data after furanone-C30 exposure. Both increases and decreases in relative-pathogenicity were observed in 2/8 isolates when exposed to F-DPD but this did not occur in EC958.

Conclusion

Quorum sensing inhibitors (QSIs) are a novel class of anti-biofilm agents that due to their site specific nature and low level cytotoxicity are being widely considered as promising anti-infective medical device coating agents. However unlike broad-spectrum antimicrobials such as biocides, their long-term effects remain largely unknown. Here we report, the impact of adaptation towards three QSIs in a panel of UPEC. Planktonic susceptibility (MIC and MBC) increased for cinnamaldehyde and furanone-C30 whilst remaining unchanged for F-DPD. Changes in susceptibility were correlated to reductions in cellular efflux and an increase in cell wall permeability. Biofilm-formation and susceptibility frequently decreased or remained unchanged after long-term QSI exposure possibly due to a downregulation of motility and chemotaxis associated systems for cinnamaldehyde and furanone-C30. Relative-pathogenicity largely increased for cinnamaldehyde exposed isolates whilst decreasing after Furanone-C30

exposure and showing a strain specific response for F-DPD. Concerningly, we observed the emergence of antibiotic resistance after QSI exposure in a 9/63 UPEC isolates. These data highlight that in order to understand the long-term effects of anti-virulence strategies such as QSIs multiple phenotypic effects need to be assessed in relevant bacterial pathogens.

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Transparency declarations

None to declare

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Figure 1: Biofilm-formation in QSI adapted UPEC

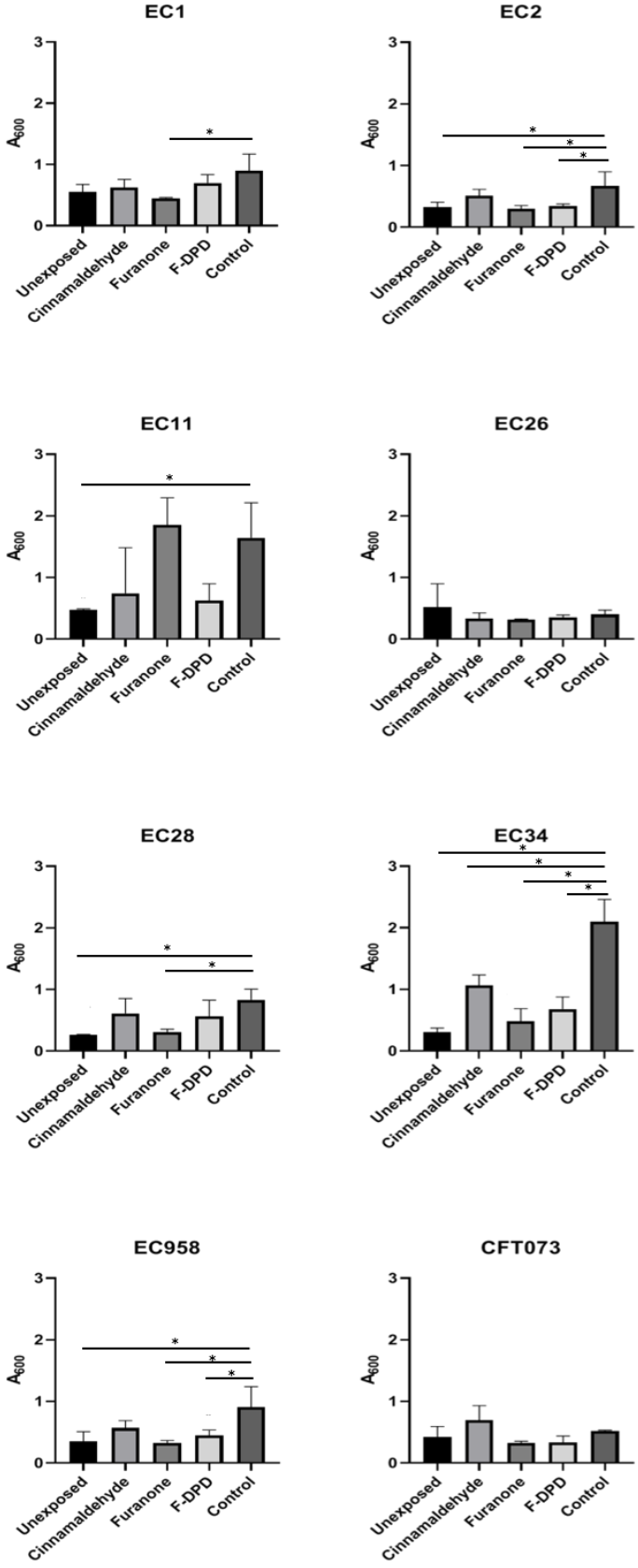
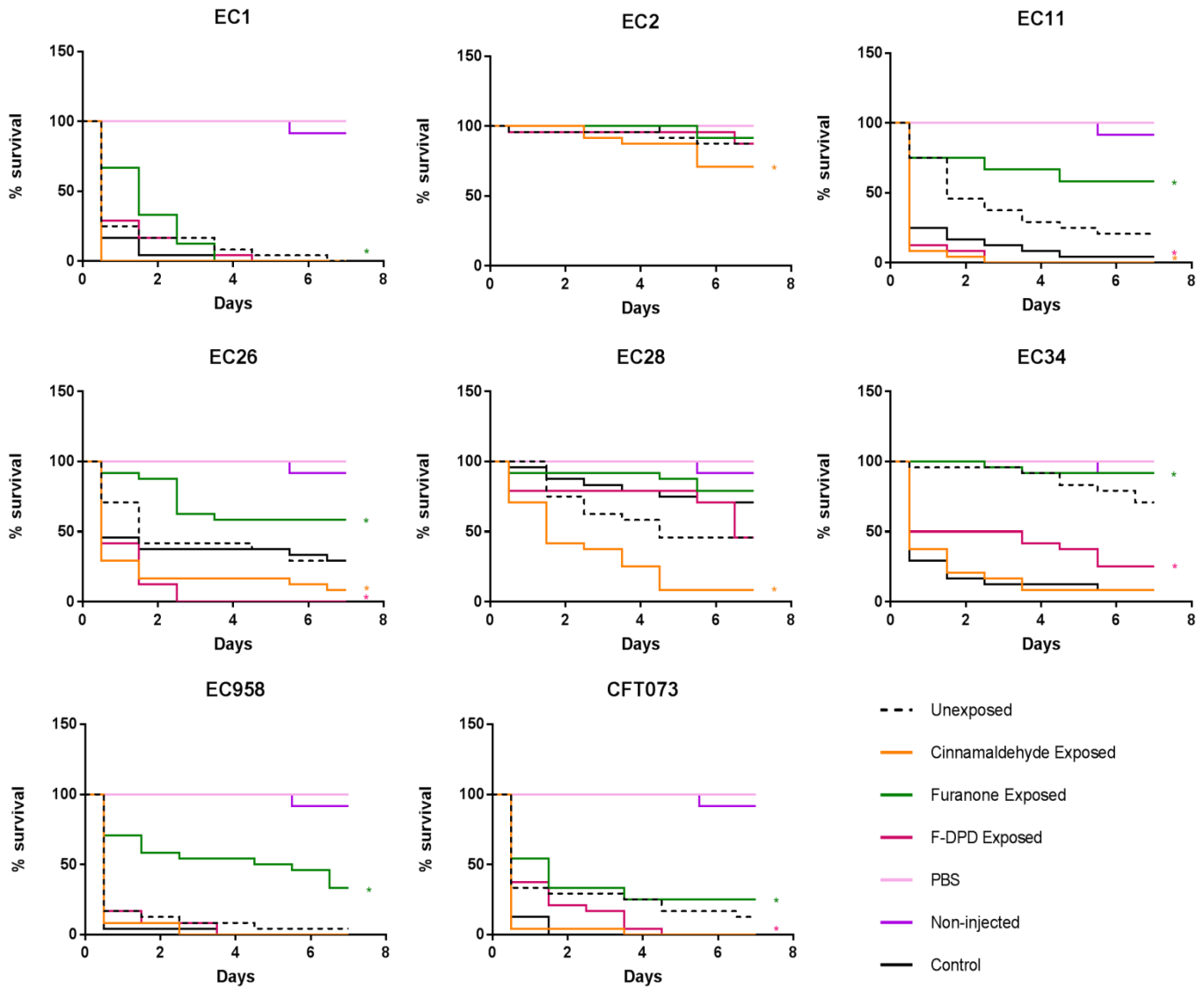


Figure 1: Crystal violet biofilm assay indicating the effect of long-term QSI exposure on biofilm-formation in eight isolates of UPEC. Data show the mean absorbance (A_{600}) representative of biofilm-formation for the following isolates; Unexposed parental isolate (P0) that has not undergone passage. Cinnamaldehyde, furanone-C30 or F-DPD exposed isolate (P12): which has undergone 12 passages in the present of named QSI. Control (C12) which has undergone 12 passages on a QSI free media. Data represent samples taken from two biological replicates each with four technical repeats. For data that varied between replicates, SDs are given as error bars. Significance was determined using ANOVA with a post hoc Tukey analysis; * $p \leq 0.05$ when comparing the unexposed isolate (P0) or a QSI passaged isolate to the respective control C12.

571 Figure 2: Relative-pathogenicity of QSI adapted UPEC



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573 Figure 2: *G. mellonella* survival curves for larvae injected with QSI-exposed and -unexposed
574 UPEC. Control data from non-injected larvae, larvae injected with PBS alone, and larvae
575 injected with control isolates passaged on a QSI free media (Control) are also shown. *
576 indicates a significant difference in pathogenicity when comparing QSI adapted isolates to the
577 respective passaged control strain ($p \leq 0.05$, log-rank reduction test n=24).

Table 1: Minimum inhibitory concentrations for UPEC before and after QSI exposure.

	Cinnamaldehyde			Furanone-C30			F-DPD		
Isolate	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**
EC1	250	**125**	500	125	**125**	500	125	125	125
EC2	250	**250**	500	125	**125**	375 (144)	125	125	125
EC11	250	**250**	500	125	**125**	375 (144)	62.5	125	125
EC26	250	**125**	500	125	**125**	313 (125)	62.5	125	125
EC28	250	**250**	500	125	**125**	500	62.5	125	125
EC34	250	**250**	500	125	**125**	313 (125)	62.5	125	125
EC958	250	**250**	500	125	**125**	375 (144)	62.5	125	125
CFT073	250	**250**	500	125	**125**	250	125	250	250

Minimum inhibitory concentrations (mg/L) for UPEC before exposure (P0), after 12 passages in the presence of each QSI (P12) or after 12 passages in a QSI free environment (C12). Data represent mean MICs taken from three biological replicates each with two technical repeats. SDs are given in parentheses. Data ≥ 2-fold-change in susceptibility are highlighted in bold when comparing the QSI passaged isolates to the respective passaged control (C12).

591 Table 2: Minimum bactericidal concentrations for UPEC before and after QSI exposure.

	Cinnamaldehyde			Furanone-C30			F-DPD		
Isolate	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**
EC1	1000	**500**	1750 (500)	250	**250**	500	250	500	500
EC2	1000	**1000**	2000	250	**125**	500	250	500	500
EC11	250	**1000**	2000	125	250	437.5 (125)	250	500	500
EC26	250	**1000**	2000	125	**125**	375 (144)	250	250	250
EC28	250	**1000**	2000	250	**125**	500	250	500	500
EC34	250	**1000**	2000	125	**125**	312.5 (125)	250	**250**	125
EC958	250	**1000**	2000	250	250	375 (144)	250	500	500
CFT073	250	**250**	1250 (500)	125	**125**	250	250	500	500

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593 Minimum bactericidal concentrations (mg/L) for UPEC before exposure to QSI (P0), after 12
594 passages in the presence of each QSI (P12), and after 12 passages in a QSI free environment
595 (C12). Data represent mean MBCs taken from three biological replicates each with two
596 technical repeats. SDs are given in parentheses. Data ≥ 2-fold-change in susceptibility are
597 highlighted in bold when comparing the QSI passaged isolates to the respective passaged
598 control (C12).

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Table 3: Minimum biofilm eradication concentrations for UPEC before and after QSI exposure.

	Cinnamaldehyde			Furanone-C30			F-DPD		
Isolate	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**
EC1	250	**250**	1000	250	250	312.5 (125)	500	500	500
EC2	250	**250**	1000	250	250	437.5 (125)	250	250	250
EC11	250	**250**	500	125	**125**	437.5 (375)	62.5	15.6	15.6
EC26	250	**250**	1000	125	**250**	500	250	250	250
EC28	250	**250**	1000	250	**125**	500	500	250	250
EC34	250	**250**	1000	125	**93.75 (44.2)**	187.5 (72.2)	62.5	62.5	62.5
EC958	250	**250**	1000	250	**125**	500	500	500	500
CFT073	250	**250**	500	250	**125**	250	500	500	500

Minimum biofilm eradication concentrations (mg/L) for UPEC before exposure to QSI (P0), after 12 passages in the presence of each QSI (P12), and after 12 passages in a QSI free environment (C12). Data represent mean MBECs taken from three biological replicates each with two technical repeats . SDs are given in parentheses. Data ≥ 2-fold-change in susceptibility are highlighted in bold when comparing the QSI passaged isolates to the respective passaged control (C12).

Table 4: Antibiotic susceptibility of UPEC before and after QSI exposure

Antibiotic	Exposure	EC1	EC2	EC11	EC26	EC28	EC34	EC958	CFT073
Trimethoprim Sulfamethoxazole	Unexposed (P0)	14.4 (1.8) S	0 R	0 R	0 R	0 R	0 R	0 R	15.3 (1) S
	Control (C12)	14.5 (0.8) S	0 R	0 R	0 R	0 R	0 R	0 R	15.1 (0.8) S
	Cinnamaldehyde (P12)	14.3 (0.8) S	11 (5.5) R	0 R	0 R	0 R	0 R	0 R	**12.8 (1.9) I**
	Furanone-C30 (P12)	14.2 (1.5) S	**11.3 (1.4) I**	0 R	0 R	0 R	0 R	0 R	**13 (3.5) I**
	F-DPD (P12)	29.7 (0.5) S	**29 (0.6) S**	0 R	3.7 (4) R	0 R	0 R	0 R	36.7 (0.8) S
Nitrofurantoin	Unexposed (P0)	20.3 (0.3) S	20.7 (0.3) S	23.7 (0.3) S	21.2 (1.5) S	19.7 (0.5) S	16.3 (1.2) S	20.4 (1.4) S	18 (0.6) S
	Control (C12)	20.5 (0.2) S	20.3 (0.5) S	22.3 (0.5) S	21.3 (1.3) S	18.1 (0.4) S	16.1 (0.8) S	20.2 (0.5) S	18.2 (0.7) S
	Cinnamaldehyde (P12)	23 (1) S	22.3 (1.5) S	26.7 (1.2) S	25 (1) S	20.3 (4.7) S	18 (3) S	20.7 (0.6) S	22 (1) S
	Furanone-C30 (P12)	24 (1) S	24.7 (0.6) S	22.3 (0.6) S	21.7 (0.6) S	18.3 (2.1) S	17.3 (0.6) S	19.7 (1.5) S	17.7 (2.3) S
	F-DPD (P12)	22.3 (0.6) S	24.7 (0.6) S	23.7 (1.2) S	26.3 (2.5) S	19.3 (0.6) S	18.7 (1.5) S	21.7 (2.1) S	19.7 (0.6) S
Ciprofloxacin	Unexposed (P0)	33 (0.6) S	0 R	0 R	0 R	25 (0.9) I	0 R	0 R	35 (1.3) S
	Control (C12)	34.2 (0.4) S	0 R	0 R	0 R	25 (0.4) I	0 R	0 R	34.2 (1.2) S
	Cinnamaldehyde (P12)	35.5 (0.8) S	0 R	0 R	0 R	**29 (1.3) S**	1.7 (4.1) R	0 R	33.3 (1) S
	Furanone-C30 (P12)	29.9 (1) S	0 R	0 R	0 R	25 (2.5) I	0 R	0 R	32.2 (1.2) S
	F-DPD (P12)	33 (1.3) S	0 R	0 R	0 R	**27.5 (1.4) S**	0 R	0 R	47.7 (2.5) S
Gentamicin	Unexposed (P0)	26 (0.5) S	27.7 (0.3) S	25.5 (0.6) S	14.3 (1.2) I	18.2 (1) S	16.5 (0.5) I	26 S	24.8 (0.4) S
	Control (C12)	24.3 (1.2) S	23.7 (1.1) S	24.9 (0.3) S	14.5 (0.8) I	18.0 (0.7) S	15.6 (0.7) I	25 (0.8) S	23.7 (0.2) S
	Cinnamaldehyde (P12)	20.2 (0.8) S	20.2 (1) S	20.2 (1) S	**12.3 (0.8) R**	**15 I**	14.3 (0.8) I	20 S	21.8 (1.8) S
	Furanone-C30 (P12)	19 (1.1) S	19.8 (0.4) S	20 S	**11 (0.9) R**	**14.5 (0.8) I**	**13.3 (1.5) R**	20.7 (1.6) S	23.9 (2.1) S
	F-DPD (P12)	17.5 (0.5) S	19 (0.6) S	19 (0.9) S	**13.7 (0.5) R**	**13.3 (0.8) R**	14.3 (0.8) I	19.2 (1) S	23 (1.7) S

Data show the mean antibiotic inhibition zones (mm) for UPEC before and after QSI exposure (mm) and represent samples taken from three separate experiments each with two technical replicates. For data that varied between replicates, SDs are given in parentheses. S = Sensitive, I =

Intermediate, R = Resistant, as defined by BSAC breakpoint. Where isolates changed from susceptible to intermediate or resistant after QSI exposure or from resistant to intermediate or susceptible this is highlighted in bold.

Table 5: Variant Analysis of QSI adapted EC958 genomes.

Product	Locus Tag	Position	Mutation Type	Protein ID	Isolate
Manganese efflux pump	*mntP*	281462	Silent	WP_001296134.1	Fur
RNA chaperone	*proQ*	290547	Nonsense	WP_000431388.1	Cin
DUF2931 family protein	21883_Unexposed_00512	1165	Missense	WP_059319623.1	Cin, DPD, Fur, Con
DUF2931 family protein	21883_Unexposed_00512	1184	Silent	WP_059319623.1	Cin, DPD, Fur
DUF2931 family protein	21883_Unexposed_00512	1232	Silent	WP_059319623.1	Cin
DNA polymerase III subunit Tau	*dnaX*	274099	Missense	WP 000122044 1	Cin
EntS/YbdA MFS transporter	21883_Unexposed_04281	302	Silent	WP_113417725.1	Cin
EntS/YbdA MFS transporter	21883_Unexposed_04281	1844	Silent	WP_113417725.1	Cin, Fur, Con

Effects of long-term exposure to QSIs on the genome sequence in EC958, data shows variant analysis of QSI exposed isolates (P12) and control passaged isolate (C12) compared to unexposed parent strain (P0).

Table 6: Significant changes in biological function in QSI adapted EC958.

GO biological process complete	Number of genes	Change	P value	Fold enrichment
Cinnamaldehyde				
Locomotion	25	-	1.01×10^{-6}	3.51
Chemotaxis	15	-	4.39×10^{-6}	5.19
Toxin biosynthetic processes	7	+	1.76×10^{-4}	9
Reactive nitrogen species metabolic process	16	-	7.58×10^{-7}	5.76
Bacterial flagellum dependent motility	14	-	1.81×10^{-4}	3.29
Drug catabolic processes	24	+	6.41×10^{-5}	2.77
Response to stress	91	+	3.01×10^{-4}	1.49
F-DPD				
Locomotion	25	-	6.29×10^{-17}	10.86
Regulation of chemotaxis	4	-	8.8×10^{-5}	27.79
Bacterial flagellum assembly	7	-	1.12×10^{-5}	11.44
Furanone-C30				
IMP biosynthetic process	7	+	9.61×10^{-6}	12.65
Sulfate transmembrane transport	4	+	4.19×10^{-4}	15.49
Ribosomal large subunit assembly	13	-	6.72×10^{-10}	12.58
Ribosomal small subunit assembly	9	-	3.98×10^{-7}	12.20
Translation	34	-	5.08×10^{-19}	7.81
Reactive nitrogen species metabolic process	7	+	1.01×10^{-4}	7.59
Drug catabolic process	10	+	1.01×10^{-3}	3.47
Carbohydrate catabolic processes	14	+	8.45×10^{-4}	2.77
Control				
Reactive nitrogen species metabolic process	10	-	9.17×10^{-4}	3.92
Ribosome assembly	19	+	1.44×10^{-5}	3.58
Aromatic amino acid family biosynthetic process	12	+	7.13×10^{-4}	3.46
Cellular biosynthetic processes	156	+	4.92×10^{-7}	1.32
Cellular respiration	33	+	1.65×10^{-6}	2.79

Effects of long-term exposure to QSIs on the transcriptome, data shows differential gene expression in the QSI exposed isolates (P12) compared to the respective passaged controls (C12) (controlling for repeated passaging). Control data shows changes in biological function after repeated passage by comparing the unexposed isolate (P0) to the control passaged isolate (C12). RNA-sequencing was performed using three biological repeats.