Biocide exposure induces changes in susceptibility, pathogenicity and biofilm formation in Uropathogenic Escherichia coli

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Biocide Exposure Induces Changes in Susceptibility, Pathogenicity and Biofilm Formation in Uropathogenic *Escherichia coli*.

Running title: Biocide Adaptation in Uropathogenic *Escherichia coli*

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Abstract

Background: Uropathogenic *Escherichia coli* (UPEC) are a frequent cause of catheter associated urinary tract infection (CAUTI). Biocides have been incorporated into catheter-coatings to inhibit bacterial colonisation whilst ideally exhibiting low cytotoxicity and mitigating the selection of resistant bacterial populations. We compared the effects of long-term biocide exposure on susceptibility, biofilm-formation and relative-pathogenicity in eight UPEC isolates. **Methods:** Minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), minimum biofilm eradication concentrations (MBEC) and antibiotic susceptibilities were determined before and after long-term exposure to triclosan, polyhexamethylene biguanide (PHMB), benzalkonium chloride (BAC) and silver nitrate. Biofilm-formation was quantified using a crystal violet assay and relative-pathogenicity was assessed via a *Galleria mellonella* waxworm model. Cytotoxicity and resulting biocompatability index values were determined against an L929 murine fibroblast cell line. **Results:** Biocide exposure resulted in multiple decreases in biocide susceptibility in planktonic and biofilm associated UPEC. Triclosan exposure induced the largest frequency and magnitude of susceptibility decreases at MIC, MBC and MBEC, which correlated to an increase in biofilm biomass in all isolates. Induction of antibiotic-cross-resistance occurred in 6/84 possible combinations of bacteria, biocide and antibiotic. Relative-pathogenicity significantly decreased after triclosan exposure (5/8 isolates), increased after silver nitrate exposure (2/8 isolates) and varied between isolates for PHMB and BAC. Biocompatibility index ranked antiseptic potential as PHMB>triclosan>BAC>silver nitrate. **Conclusion:** Biocide exposure in UPEC may lead to reductions in biocide and antibiotic susceptibility, changes in biofilm-formation and alterations relative-pathogenicity. These data indicate the multiple consequences of biocide adaptation that should be considered when selecting an anti-infective catheter-coating agent.

Introduction

Catheter-associated urinary tract infections (CAUTI) are amongst the most commonly acquired healthcare associated infections contributing considerably to patient morbidity and posing an economic burden on healthcare service providers (1). Complications associated with catheterisation often arise due to contamination of the catheter surface with uropathogenic *Escherichia coli* (UPEC) during catheter insertion, leading to the formation of bacterial biofilms and subsequent infection. Patients undergoing long-term catheterisation are at a particular risk of acquiring CAUTI, with studies indicating a 5-8% increase in the risk of developing bacteriuria for every day that the catheter remains inserted (2). The majority of patients will exhibit bacteriuria after four weeks of catheterisation, potentially leading to further complications such as pyelonephritis and septicaemia (2, 3).

Bacterial biofilms are often recalcitrant to antimicrobial chemotherapy and to the actions of the host immune system, making biofilm associated infections such as CAUTIs difficult to treat (4). Biofilms show decreased susceptibility to antibiotics, partially due to the shielding effect of the extracellular polymeric substance (EPS) encasing the bacterial cells (5), the low metabolic activity of the cells within the biofilm (6) and the activity of membrane-bound efflux pumps that actively expel antimicrobial compounds from the bacterial cell (4). Furthermore, antibiotic-resistance genes are frequently transferred between bacteria within a biofilm by horizontal gene transfer allowing the dissemination of resistance through a bacterial population (7). Antibiotic treatment of CAUTIs is therefore often ineffective due to the recalcitrance of the biofilm in addition to the increasing prevalence of antibiotic resistant uropathogens (8). There is considerable interest in developing anti-infective catheter coatings that are refractory to microbial colonisation and subsequent biofilm formation in an attempt to prevent the establishment of CAUTI.

Biocides are broad-spectrum antimicrobial chemicals that inhibit the growth of, or kill microorganisms (9). Biocide coated urinary catheters have been developed incorporating biocides such as silver nitrate and nitrofurazone that are eluted from the surface of the catheter providing an antimicrobial gradient and a potential selective pressure for biocide resistant populations of bacteria (10). Current clinical trial data has highlighted the limited antimicrobial efficacy of silver-impregnated catheters when compared to those without an antimicrobial coating, whilst nitrofurazone-containing coatings have been shown to exhibit only short-term antimicrobial activity and may therefore be ineffective in patients undergoing long-term catheterisation (11, 12). This has fuelled the search for further anti-infective coating agents that display broad-spectrum activity which is maintained after prolonged use.

Long-term exposure of certain bacterial species to biocides may cause the induction of biocide insusceptibility either through the selection of intrinsically resistant mutants or through induced phenotypic adaptations, bringing into question the long-term antimicrobial activity of various biocide containing coatings (13). Concerns have also been raised that long-term biocide exposure may promote cross-resistance to antibiotics through the acquisition of mutations in shared target sites or through the activation of broad-range defence mechanisms (14), such as increased cellular efflux activity (15) or decreased cell permeability (16). It can, however, be argued that whilst long-term biocide exposure may lead to reductions in biocide or antibiotic susceptibility in bacteria, these reductions are small and would not impact on the susceptibility of bacteria to the concentrations of biocide used in practice. Furthermore, such changes in biocide susceptibility may be accompanied with functional deficits impacting biofilm formation, pathogenicity and competitive fitness in bacteria (17). Therefore in order to develop an effective anti-infective catheter coating the multiple long-term effects of the biocide used within the coating must be taken into consideration.

Whilst previous investigations have evaluated the impact of long-term biocide exposure on the antimicrobial susceptibility of many clinically relevant bacteria, there is no current investigation into the multiple phenotypic consequences that may occur due to long-term biocide exposure in UPEC. The current study therefore aims to quantify the effects of long-term biocide exposure in eight UPEC isolates. The commonly used biocides PHMB, triclosan, BAC and silver nitrate were evaluated for their long-term antibacterial and anti-biofilm activity and their potential to induce antibiotic cross-resistance. The impact that biocide exposure has on bacterial relative pathogenicity was assessed using a *Galleria mellonella* waxworm model and the biocides antiseptic potential was determined via calculating cytotoxicity in an L929 murine fibroblast cell line allowing the determination of a biocompatibility index value (18).

Methods

Bacteria and chemicals. Six UPEC clinical isolates (EC1, EC2, EC11, EC26, EC28 and EC34) previously isolated from urinary tract infections (Stepping Hill Hospital, Stockport, UK) and two laboratory characterised UPEC strains EC958 and CFT073 were used in the investigation. Bacteria were cultured on Muller-Hinton agar (MHA; Oxoid, UK) and Muller-Hinton broth (MHB; Oxoid, UK) and incubated aerobically at 37 °C for 18 h, unless otherwise stated. Biocides were formulated as follows: triclosan solubilised in 5% (v/v) ethanol. Polyhexamethylene biguanide (PHMB) (LONZA, Blackley, UK), benzalkonium chloride (BAC) and silver nitrate were prepared at 1 mg/ml in water and filter sterilised prior to use. All chemicals were purchased from Sigma–Aldrich (Poole, UK) unless otherwise stated.

Long-term exposure of bacteria to biocides. Bacteria were repeatedly exposed to biocides using an antimicrobial gradient plating system adapted from McBain *et al* (19). In

brief, 100 µl of a 5 × MBC concentration solution of biocide was added to an 8 x 8 mm well in the centre of a 90 mm agar plate. Bacterial pure cultures were radially inoculated in duplicate from the edge of the plate to the centre, prior to incubation for 2 days aerobically at 37°C. Biomass from the inner edge of the annulus of bacterial growth representative of the highest biocide concentration at which growth could occur was removed and used to inoculate a new biocide containing plate, as outlined above. This process was repeated for 12 passages. Control isolates passaged 12 times on biocide free media were also included. Bacteria were archived at -80 °C before and after biocide passage for subsequent testing.

Minimum inhibitory and minimum bactericidal concentration. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined as described previously (17). In brief, 2 × 5 ml overnight cultures of test bacteria were prepared in MHB prior to overnight incubation (18-24 h) at 37ºC and 100 rpm. Cultures were diluted to an OD_{600} of 0.008 in 20 ml of sterile MHB to produce a bacterial inoculum for biocide susceptibility testing. Doubling dilutions (150 µl) of each test biocide were prepared in sterile MHB in a 96-well microtiter plate prior to addition of bacterial inoculum (150 µl). Plates were incubated overnight (18-24 h) at 37ºC and 100 rpm. The MIC was defined as the lowest concentration of biocide for which growth was completely inhibited (viewed as 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 onto Muller Hinton Agar (MHA) in triplicate. The plates were incubated statically for 18-24 h at 37ºC. The lowest test concentration for which visible bacterial growth was completely inhibited was deemed the MBC.

Minimum biofilm eradication concentration. Minimum biofilm eradication concentrations were determined using the Calgary biofilm device (CBD) as described previously (20). Briefly, 2 × 5 ml overnight cultures of test bacteria were prepared in MHB

and were incubated for 18-24 h at 37ºC and 100 rpm before being diluted to an OD_{600} of 0.008 in MHB to create a bacterial inoculum for biofilm susceptibility testing. 100 µl of bacterial inoculum was added to each well of the CBD base, plates were incubated at 37ºC for 48 h to allow biofilm formation on the pegs. Doubling dilutions of biocides were prepared in sterile broth across a 96-well microtiter plate. Biofilms were exposed to antimicrobial compounds and incubated for 24 h at 37°C and 100 rpm. After incubation, the pegged lid was transferred to a 96-well plate containing 200 µl of sterile broth and was incubated for 24 h at 37°C and 100 rpm. MBEC was defined as the lowest concentration of biocide for which re-growth was completely inhibited (viewed as turbidity relative to a sterile negative control) indicating complete biofilm eradication.

Crystal violet bacterial attachment assay. 2 × 5 ml overnight cultures of test bacteria were diluted to an OD_{600} of 0.008 in MHB after incubation for 18-24h at 37º C and 100 rpm. 150 µl of diluted overnight bacterial culture was added to the wells of a sterile 96-well microtiter plate. Plates were incubated statically for 48 h at 37ºC. Media was removed from wells and replaced with 180 µl of crystal violet solution. The plate was left at room temperature for 30 minutes, crystal violet solution was decanted and the wells were rinsed with 3 × 200 µl of PBS prior to drying for 1 h at 37ºC. The remaining crystal violet was solubilised in 250 µl of 100% ethanol. The A_{600} of the solubilised crystal violet solution was determined and compared to a sterile MHB negative control.

***Galleria mellonella* pathogenicity assay.** The pathogenesis model was adapted from that of Peleg *et al* (21). Final larval-stage *G. mellonella* (Live Foods Direct, Sheffield, UK) were stored in the dark at 4°C for up to 7 days, before randomly assigning 24 to each treatment group and incubating at 37°C for 30 min. Overnight suspensions of *E. coli* were pelleted via centrifugation at 13,000 rpm, washed twice in 1 ml of PBS and then diluted appropriately to achieve an OD_{600} of 0.1 (5×10^5 - 8×10^5 CFU/ml, as confirmed by colony

169 counts on MHA). Aliquots of each suspension (5 µl) were injected into the hemocele of each
170 larva via the last left proleg using a Hamilton syringe. Larvae were incubated in a petri dish at
171 37°C and the number of surviving individuals was recorded daily. An untreated group and a
172 group injected with sterile PBS were used as additional controls. The experiment was
173 terminated when at least two individuals in a control group had died or after 7 days of
174 incubation. Two independent bacterial replicates were used to inoculate 24 caterpillars (12
175 per replicate) and significance in death rate was calculated using a log-rank reduction test
176 ($p \leq 0.01$).

177 **Biocompatibility index**. Calculation of biocompatibility index (BI) was performed as
178 described by Muller and Kramer (18). To determine cytotoxicity, Neutral Red (NR) (3-
179 amino- 7-dimethylamino-2-methylphenazine hydrochloride) assays and MTT [3-(4,5-
180 dimethylthiazol-2-yl)-2, 5-diphenyltetra-zolium bromide] assays were performed on an L929
181 cell line to establish IC_{50}. Procedures for the NR assay and the MTT test have been described
182 in detail elsewhere (18). The bacterial quantitative suspension tests were done in accordance
183 with the guidelines for testing disinfectants and antiseptics of the European Committee for
184 Standardization (22). Suspension tests were performed in the presence of serum to determine
185 the rf value, defined as the concentration of biocide that achieved a reduction in bacterial load
186 of at least $3\log_{10}$ (99.9%). Suspension tests were conducted as follows, overnight bacterial
187 cultures were diluted to 10^8-10^9 CFU/ml as determined by colony counts on MHA. 15 µl
188 aliquots of inoculum were then transferred into 135 µl of biocide containing cell culture
189 medium prior to incubation for 30 min at 37ºC. For PHMB, BAC, and triclosan, the biocide
190 was subsequently inactivated by transfer of 15 µl of the suspension into 135 µL of TSHC
191 (3% (w/v) Tween 80, 3% (w/v) saponin, 0.1% (w/v) histidine and 0.1% (w/v) cysteine).
192 Silver nitrate was inactivated using TLA-thio (3% [w/v] Tween 80, 0.3% lecithin from soy
193 bean, 0.1% [w/v] histidine and 0.5% [w/v] sodium thiosulphate). After 30 min of inactivation,

5 µl aliquots were spot plated onto MHA in triplicate. The plates were incubated statically for 18-24 h at 37ºC and CFU/ml was determined. The lowest test concentration which achieved at least a $3\log_{10}$ (99.9%) reduction in bacterial load was deemed the rf value. BI is calculated as IC50/rf for each combination of biocide and isolate and indicates the antiseptic potential of the test compound.

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 British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method for antimicrobial susceptibility testing (23).

Determination of mutation rate frequency. Mutation rate frequency was determined as described by Miller *et al* (24) In brief, 100 µl aliquots of diluted overnight culture obtained from single bacterial colonies were plated onto antibiotic free MHA plates and MHA plates containing 50µg/ml rifampicin in triplicate. Plates were incubated for 24h at 37ºC prior to determination of viable count. Mutation frequencies were expressed as the number of resistant mutants recovered as a fraction of total viable bacteria.

Results

Biocide susceptibility of UPEC in planktonic and biofilm states. MIC, MBC and MBECs were determined for all test isolates before (P0) and after repeated passage either in the absence (C12) or presence of a specific biocide (P12) (Tables 1-3). Change in biocide susceptibility after exposure was calculated as fold-change relative to the control (C12, Table S1). Data indicates both the frequency of susceptibility change (≥2 fold) and the average magnitude of susceptibility change for each biocide.

In terms of MIC, after repeated biocide exposure there was a ≥2 fold increase in 4/8 isolates for BAC, 8/8 for silver nitrate and 8/8 for triclosan compared to the respective bacteria passaged in a biocide free environment (Table 1). In contrast 4/8 isolates showed a ≥2 fold decrease in MIC after exposure to PHMB. The average fold-change for MIC (C12 to P12) across the test panel of UPEC was 1.5 for BAC, 0.7 for PHMB, 2 for silver nitrate and 807.1 for triclosan (Table S1). For MBC in the biocide exposed isolates (Table 2) there was a ≥2 fold increase in 4/8 isolates after BAC exposure, 8/8 for silver nitrate and 5/8 for triclosan. In contrast 1 isolate showed a decrease in MBC after PHMB exposure. The average fold change in MBC after biocide exposure was 1.5 for BAC, 0.8 for PHMB, 3.8 for silver nitrate and 5.4 for triclosan (Table S1). In terms of MBEC (Table 3), after repeated biocide exposure there was a ≥2 fold increase in 7/8 isolates for BAC, 8/8 for PHMB and 8/8 for triclosan. Silver nitrate exposure led to a 1 increase in MBEC and 1 decrease. The average fold change in MBEC after biocide exposure was 4.5 for BAC, 29.2 for PHMB, 832.7 for triclosan and 7.8 for silver nitrate (Table S1). We observed a number of changes in MIC, MBC and MBEC after the passage of bacteria solely in a biocide free-environment when compared to the unpassaged parent isolate. We did not, however see any incidence of a control passaged isolate (C12) exhibiting a significantly higher MIC, MBC or MBEC ($P<0.05$) than the respective biocide passaged isolate (P12) with the exception of PHMB where the biocide exposed isolates frequently exhibited a lower MIC and MBC than the unexposed parent strain and the control passaged isolate subsequently matched the susceptibility of the parent strain.

The impact of biocide exposure on UPEC biofilm formation. Biofilm formation was determined via a crystal violet biofilm assay for each UPEC isolate before and after repeated biocide exposure and after passage in a biocide free media (Figure 1). Unexposed isolates displayed varying biofilm forming capabilities prior to biocide exposure with EC2 showing the highest level of biofilm formation followed by

EC1>CFT073>EC11>EC28>EC34>EC26 and EC958. When repeatedly exposed to triclosan, all isolates (with the exception of CFT073) demonstrated a significant (ANOVA $p \leq 0.05$) increase in biofilm formation relative to the respective control. All isolates demonstrated a significant increase in biofilm formation after BAC exposure with the exception of EC2. For PHMB and silver nitrate, EC1 showed a significant increase in biofilm formation after repeated exposure to either biocide. PHMB exposure also induced decreases in biofilm formation in EC2 and CFT073. Differences in biofilm formation were determined to be irrespective of growth rate as we did not observe any significant (ANOVA $p<0.05$) change in growth rate or overall growth productivity when in binary culture (Figure S1).

Relative pathogenicity of UPEC after long-term biocide exposure. A *G. mellonella* waxworm model was used to determine relative pathogenicity in UPEC isolates (Figure 2). Data indicate that prior to biocide exposure, EC2 was the least pathogenic and EC1 and EC958 were the most pathogenic isolates. PHMB exposure induced significantly (log-rank $p \leq 0.05$) decreased relative pathogenicity in 3/8 isolates (EC11, EC34 and EC958) and a significant increase in pathogenicity for EC2 when compared to the respective control isolate (C12). BAC exposure induced significantly decreased pathogenicity in 6/8 isolates (EC1, EC11, EC26, EC28, EC34 and EC958) and significantly increased pathogenicity in EC2. Silver nitrate was the only biocide to only induce significant increases in pathogenicity which occurred in 2/8 isolates (EC11 and EC28) and triclosan was the only biocide to induce only significant decreases in pathogenicity which occurred in 5/8 isolates (EC11, EC26, EC34, EC958 and CFT073).

Changes in antibiotic susceptibility after biocide exposure. Isolates were classed as resistant or sensitive to each antibiotic as defined by BSAC breakpoints (23). Antibiotic susceptibility was determined for UPEC isolates before and after exposure to each biocide (Table 4). Data indicate that PHMB exposure induced CFT073 to become resistant to

trimethoprim sulfamethoxazole and EC26 to become resistant to gentamicin. Exposure to triclosan induced nitrofurantoin resistance in EC958 and ciprofloxacin resistance in EC2. Silver nitrate exposure induced EC2 to become resistant to ciprofloxacin as did BAC exposure. There were cases where isolates that were initially resistant to trimethoprim sulfamethoxazole became more susceptible after biocide exposure. This occurred in EC2 after exposure to PHMB, BAC, or silver nitrate and in EC11 after exposure to triclosan or BAC. This was also observed in EC11 for ciprofloxacin after triclosan exposure and CFT073 after BAC, triclosan or silver nitrate exposure.

Biocompatibility Index. Cytotoxicity data for the four biocides against an L929 cell line are shown in Table 5, rf values, indicating antimicrobial activity, and the corresponding BI values, highlighting the antiseptic potential of the compounds, are shown in Table 6. The order of cytotoxicity in relation to the biocide concentration was silver nitrate>PHMB>BAC>triclosan. The only isolate for which an rf value could be determined for silver nitrate was CFT073 as the rf values for the other isolates exceeded the maximum solubility of the biocide. Similarly, an rf value could not be determined in EC28 and CFT073 for triclosan as the rf value was greater than the highest achievable test concentration. BI values for the eight isolates were averaged for each biocide and the final ranked order of BI was PHMB>triclosan>BAC>silver nitrate indicating the antiseptic potential of the biocides.

Mutation rate frequency in UPEC isolates. Mutation rate frequency was determined with regards to rifampicin resistance. We observed rifampicin resistant mutants from all UPEC isolates (Table 7). Mutation frequencies varied from 1.7×10^{-8} for CFT073 up to 3×10^{-7} for EC2 with an overall mutation frequency rank order of EC2>EC28>EC11>EC1>EC34>EC958>EC26>CFT073.

Discussion

The current investigation aimed to explore the phenotypic changes that occur in genetically mixed populations of UPEC as a result of long-term biocide exposure. Susceptibility of eight UPEC isolates to a panel of test biocides was determined in planktonic and biofilm states before and after long-term biocide exposure. Changes that biocide exposure had on biofilm formation, relative pathogenicity and antibiotic susceptibility were assessed. Furthermore, cytotoxicity and the corresponding BI values were determined for each biocide against an L929 murine fibroblast cell line indicating the antiseptic potential of the test agents.

Biocide exposure induces changes in antimicrobial susceptibility in planktonic UPEC. The data in this investigation highlights that long-term exposure to biocides may influence biocide susceptibility in UPEC. Bacterial susceptibility to biocides can be markedly affected by structural variations in the bacterial cell that (i) impact attraction of the biocide to the cell (16) (ii) lead to changes in cell permeability to the biocide (25) and (iii) cause modification in efflux activity allowing the bacteria to expel the biocide from the cell (26). These modifications may account for some of the changes in biocide susceptibility observed in the current study, however the exact mechanisms that govern each specific adaptation depends upon a multitude of factors inherent to both the particular biocide and the bacterium (16). Furthermore, previous studies have indicated that biocide exposure in bacteria may result in reversible phenotypic adaptations that occur as a consequence of temporary changes in gene expression, for instance the induction of stress responses (27). In contrast, other investigations highlight that biocide exposure may lead to the selection of biocide resistant mutants with stable phenotypes that do not revert in the absence of the biocide (28). This may reflect diversity within the mechanisms of action of biocides particularly with regards to target site specificity. Bacterial exposure to target site specific biocides such as triclosan readily appears to lead to the selection of mutations in target enzyme FabI (28) whilst induced insusceptibility towards membrane active compounds such as biguanides (PHMB)

and quaternary ammonium compounds (BAC) is often associated with the induction of stress responses (29, 30).

In terms of initial antimicrobial efficacy, silver nitrate demonstrated the lowest activity against planktonic UPEC when compared to other test biocides at MIC and MBC. We observed a high frequency of small magnitude decreases (≤2-fold) in silver nitrate susceptibility after long-term exposure resulting in comparatively high MIC and MBC values. Silver is widely considered as an effective anti-infective urinary catheter coating agent and is used in currently marketed anti-infective urinary catheters (31). However, previous investigations have also documented the selection of silver resistance in Gram negative pathogens (32) including *E. coli* and other invasive Enterobacteriaceae (33). This resistance has been correlated to increased efflux activity (34) or a loss of outer membrane porins (35) thereby decreasing cell permeability, which may explain the induced reductions in silver nitrate susceptibility observed in our UPEC isolates.

PHMB exposure induced a high frequency of small magnitude (≤2-fold) increases in susceptibility in planktonic UPEC at MIC and MBC. Previous data indicate that changes in bacterial susceptibility in response to membrane active compounds, such as biguanides, is usually attributed to alterations in the structural integrity of the bacterial cell envelope impacting cell permeability, modifications in the structure of LPS interfering in electrostatic interactions between the cationic biocide and cell envelope and due to increased cellular efflux activity, expelling the biocide from the cell (27), these mechanisms of resistance are in contrast with the data in the current investigation. Whilst other studies have also highlighted increases in PHMB susceptibility in bacteria after long-term exposure the underlying mechanisms that govern this adaptation remains unknown. It has been suggested that long-term exposure to certain biocides in bacteria may result in cumulative cellular damage and a resulting loss of fitness increasing bacterial susceptibility over time (13). The potential for

PHMB to lead to increased susceptibility in bacteria after long-term exposure is an attractive attribute when considering an antimicrobial catheter coating agent, particularly in catheters that would be required for longer term use and are therefore prone to the selection of resistant microorganisms.

Triclosan was the most potent antimicrobial before repeated biocide exposure in planktonic UPEC. However triclosan induced the largest frequency and magnitude of susceptibility decreases in MIC and MBC. Resistance of *E. coli* to triclosan has been widely documented and is believed to be due to a mutation in the target enzyme FabI (28), due to increased cellular efflux (15) and changes in the cell membrane composition that reduce permeability (36). Triclosan-impregnated catheters have demonstrated marked efficacy in *in vitro* studies (37), and show little reduction in antimicrobial activity even after long-term use (38). This may be due to the fact that whilst large susceptibility changes may occur in bacteria following triclosan exposure, as indicated in our data, the initial potency of triclosan means that the catheter maintains a high level of antimicrobial activity even after the bacteria adapt to the presence of the biocide likely due to its multi-target site mode of action.

BAC demonstrated lower initial antimicrobial activity against planktonic UPEC compared to triclosan and PHMB (MIC and MBC) and only induced minor reductions (≤2-fold) in susceptibility after long-term exposure. Changes in gene expression in BAC adapted *E. coli* have been previously identified revealing an upregulation of efflux pump membrane transporter *yhiV* and downregulation of the outer membrane porin *ompA* thereby increasing cellular efflux of BAC and reducing cell permeability towards the biocide (39).

Repeated passage of bacteria on a biocide free media occasionally led to changes in biocide susceptibility within planktonic culture, however these changes occurred at a substantially lower magnitude and frequency than those observed after biocide adaptation and were

predominantly increases in susceptibility. This potentially emphasises the fitness costs associated with repeated culture. Significantly, we did not see any reduction in biocide susceptibility when comparing the isolate passaged in the absence of biocide to the unexposed parent strain.

Biofilm formation and susceptibility in UPEC after biocide exposure. Bacteria that have adapted to the presence of biocides may exhibit further phenotypic alterations such as changes in growth rate, biofilm formation and competitive fitness, which may influence pathogenicity (13, 17). After biocide exposure several UPEC isolates in the current study exhibited significant changes in biofilm formation. Whilst this biofilm formation is a complex multifactorial process, these changes could potentially be attributed to the selection of mutants with alterations in factors involved in the establishment of biofilms, such as adhesion, EPS production or maturation.

Biocide exposure largely led to increases in biofilm formation particularly after exposure to BAC and triclosan. Of the 7 UPEC isolates that demonstrated an increase in biofilm formation after BAC exposure 6 had a corresponding increase in MBEC. All 7 isolates that increased in biofilm formation after triclosan exposure also exhibited an elevation in MBEC. PHMB exposure led to a significant decrease in biofilm formation for EC2 and CFT073 which did not correspond with decreases in MBEC, possibly indicating the recalcitrance of persister populations within the biofilm irrespective of biofilm biomass (40).

BAC adaptation has been previously correlated to an increase in biofilm biomass in *E. coli* which is believed to be due to an increase in protein and polysaccharide content within the extracellular polymeric substance (EPS) (41). This change in EPS composition may lead to reduced BAC susceptibility, as observed in our BAC adapted isolates. Yu *et al*. (42) utilised a genome-wide enrichment screen to demonstrate the genes involved in triclosan adaptation in

389 *E. coli*. Microarray analysis revealed that triclosan exposure resulted in an increase in
390 *fimDFHI* which encodes proteins involved in fimbrial biosynthesis, that have been shown to
391 be positively associated with an increase in biofilm formation (43). This may provide a
392 potential link between the increase in biofilm formation and thus resistance caused by
393 triclosan exposure in the UPEC isolates used in the current investigation.

394 **Changes in antibiotic susceptibility after biocide exposure in UPEC.** Concerns
395 have been raised that biocide exposure may induce cross-resistance to clinically relevant
396 antibiotics**.** In the current study we observed the generation of antibiotic resistance in 6 out of
397 a possible 84 combinations of bacteria, biocide and antibiotic. The biocides that induced the
398 highest number of cases of cross-resistance in a previously susceptible or intermediate isolate
399 were triclosan, which was to nitrofurantoin and ciprofloxacin, and PHMB which was to
400 trimethoprim sulfamethoxazole and ciprofloxacin. BAC and silver nitrate exposure led to one
401 observed case of cross-resistance each which was towards ciprofloxacin.

402 There have been previous reports into efflux mediated cross-resistance between antibiotics
403 and to triclosan reportedly due to upregulation of *acrAB*, encoding the AcrAB efflux pump
404 (44). Efflux pumps have also been correlated to observed cross-resistance to between
405 quaternary ammonium compounds and antibiotics in *E. coli*. Bore *et al* observed reduced
406 antibiotic susceptibility in BAC-adapted *E. coli* which also coincided with an increase in the
407 expression of *acrAB* and a downregulation in multiple outer membrane porins including
408 OmpA, OmpF and OmpT (39). Whilst there is relatively sparse evidence on the generation of
409 antibiotic cross-resistance due to PHMB exposure in bacteria, the mechanisms of uptake of
410 PHMB is similar to that of aminoglycosides involving destabilisation of the bacterial cell
411 membrane and LPS reorganisation (45). Interaction between LPS and PHMB is known to be
412 a key step in the initial interaction of the biocide with the bacterial cell in *E. coli* (31). This
413 may suggest why an induced reduction in PHMB susceptibility in our UPEC isolates also led

414 to a similar reduction in susceptibility towards gentamicin. Studies on silver resistance in *E.*
415 *coli* have revealed acquired low-level cross-resistance to cephalosporins, similarly due to
416 increased efflux and reduced porin expression (35). In this study, there were 9 cases of
417 biocide exposure eliciting increased susceptibility trimethoprim sulfamethoxazole or
418 ciprofloxacin. This occurred in 3 isolates after exposure to BAC or PHMB, in 2 isolates after
419 silver nitrate exposure and in 1 isolate after PHMB exposure. This display of "cross-
420 protection" has been noted in previous studies and has been suggested to be due to a potential
421 increase in cell permeability in response to biocide adaptation however the underlying
422 mechanisms remain unclear (17). An increase in susceptibility to clinically relevant
423 antibiotics in previously resistant uropathogens would be an extremely beneficial attribute
424 when considering a coating agent to combat the establishment of CAUTI.

425 **Biocompatibility of test biocides in an L929 cell line.** To assess the suitability of an
426 antiseptic agent both the antimicrobial activity and cytotoxicity must be considered. Silver
427 nitrate showed the highest level of cytotoxicity in an L929 cell line and the lowest
428 antimicrobial efficacy in the corresponding quantitative suspension test (rf value). Reduced
429 activity of silver when in the presence of serum has been previously attributed to binding of
430 the silver cations to the electronegative serum components, which may explain the low level
431 of antimicrobial activity in silver nitrate observed in the quantitative suspension test in the
432 current study (46). Silver ions have been demonstrated to interact with components of
433 mammalian cells including the mitochondria, nuclei, endoplasmic reticulum and the cell
434 membrane (47). Interaction of silver ions with mitochondria reportedly causes mitochondrial
435 damage and the release of reactive oxygen species (ROS) resulting in apoptosis suggesting a
436 mechanism of silver-mediated cytotoxicity (48). Whilst PHMB was shown to be the second
437 most cytotoxic biocide tested, it exhibited a relatively low rf value resulting in the highest BI
438 value out of all the test biocides. PHMB has previously shown low level cytotoxicity towards

mammalian cells, including L929 cells, which is suggested to be due to the interaction of the biocide with the mammalian cell membrane leading to membrane damage (49). BAC was the second least cytotoxic biocide tested and the showed the second highest level of antimicrobial activity in the presence of serum in the quantitative suspension tests. BAC has been shown to interact with guanine nucleotide triphosphate-binding proteins (G proteins) impacting cell signalling transduction in mammalian cells and causing DNA damage (50). Cytotoxicity data indicated triclosan to be the least cytotoxic of all the test biocides. However the rf values were high resulting in the second highest BI value. Triclosan has previously shown reduced antimicrobial efficacy in the presence of serum, this is believed to be due to the bacteria's ability to gain an exogenous supply of fatty acids from the serum, thereby bypassing the inhibitory effects of the biocide (51). Additionally, previous studies report on triclosan interference with mitochondrial respiration (52) in addition a damaging effect on the plasma membrane and induced apoptotic cell death (53) suggesting a potential mechanism of cytotoxicity.

Altered relative pathogenicity in biocide adapted UPEC. Repeated biocide exposure to silver nitrate induced an increase in relative pathogenicity in 2/8 isolates of UPEC whilst PHMB exposed isolates exhibited a decrease in pathogenicity in 3/8 and an increase in pathogenicity in 1/8 isolates respectively. A decrease in pathogenicity was observed after triclosan exposure in 5/8 isolates and in 6/8 isolates after exposure to BAC. BAC also induced an increase in pathogenicity in 1 further isolate. Triclosan exposure has previously been shown to reduce relative pathogenicity in a *G. mellonella* waxworm model in certain bacterial species (54). These pathogenicity changes were suggested to be due to changes in virulence factor production, specifically reduced DNase activity and a down-regulation in cell surface adhesins (55). It has been shown that triclosan exposure specifically downregulates genes encoding the outer membrane proteins P-fimbriae and protein X in *E.*

coli (56) which are integral for UPEC attachment to cell surfaces (57) and entry into host cells (58). Isolates of *E. coli* that have been exposed to BAC have been shown to have increased hemolysin activity and enhanced virulence (59) which may explain the increase in pathogenicity in EC2 after BAC exposure. To our knowledge there are no current studies regarding the effects of silver or PHMB exposure on bacterial virulence factor production and resulting pathogenicity.

Consequence of variance in mutation rate frequency in UPEC. Elevated mutation rates have been previously reported in *E. coli* strains (60). Furthermore, the adapting populations generated in the current investigation may lead to the selection of hypermutators due to the selective pressures created during biocide exposure. We evaluated the mutation frequencies in our parent isolates to determine whether this correlated to a higher frequency of phenotypic adaptations after biocide exposure. Mutation rate frequency was determined to be ordered EC2>EC28>EC11>EC1>EC34>EC958>EC26>CFT072. When comparing mutation rate to incidences of biocide susceptibility change (MIC, MBC and MBEC) EC11 and CFT073 showed the highest frequency of changes in biocide susceptibility whilst EC28 showed the least. We observed two cases of significant change in biofilm formation for each isolate with the exception of EC1 for which we observed four. In terms of significant changes in relative pathogenicity, EC11 demonstrated four significant changes after biocide exposure, EC34 and EC958 showed three, EC2, EC26 and EC28 showed 2 and EC1 and CFT073 showed one. With regards to changes in antibiotic susceptibility, we saw the highest number of incidences of cross-resistance towards EC2. These data indicate a potential correlation when comparing mutation rate frequency and antibiotic cross-resistance in UPEC but this trend does not extend to all aspects of phenotypic adaptation that occur as a result of biocide exposure.

Conclusion. The use of biocides for the purpose of antisepsis has led to concern over the selection of biocide resistance in clinically relevant pathogens. Here we demonstrate that long-term exposure of UPEC to commonly used biocides can result in changes in biocide susceptibility which may be accompanied by further phenotypic alterations impacting biofilm formation, antibiotic susceptibility and relative pathogenicity. The multiple consequences of bacterial adaptation towards biocides should therefore be evaluated when considering a potential anti-infective catheter coating agent.

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667 Figure 1: Biofilm formation in biocide adapted UPEC

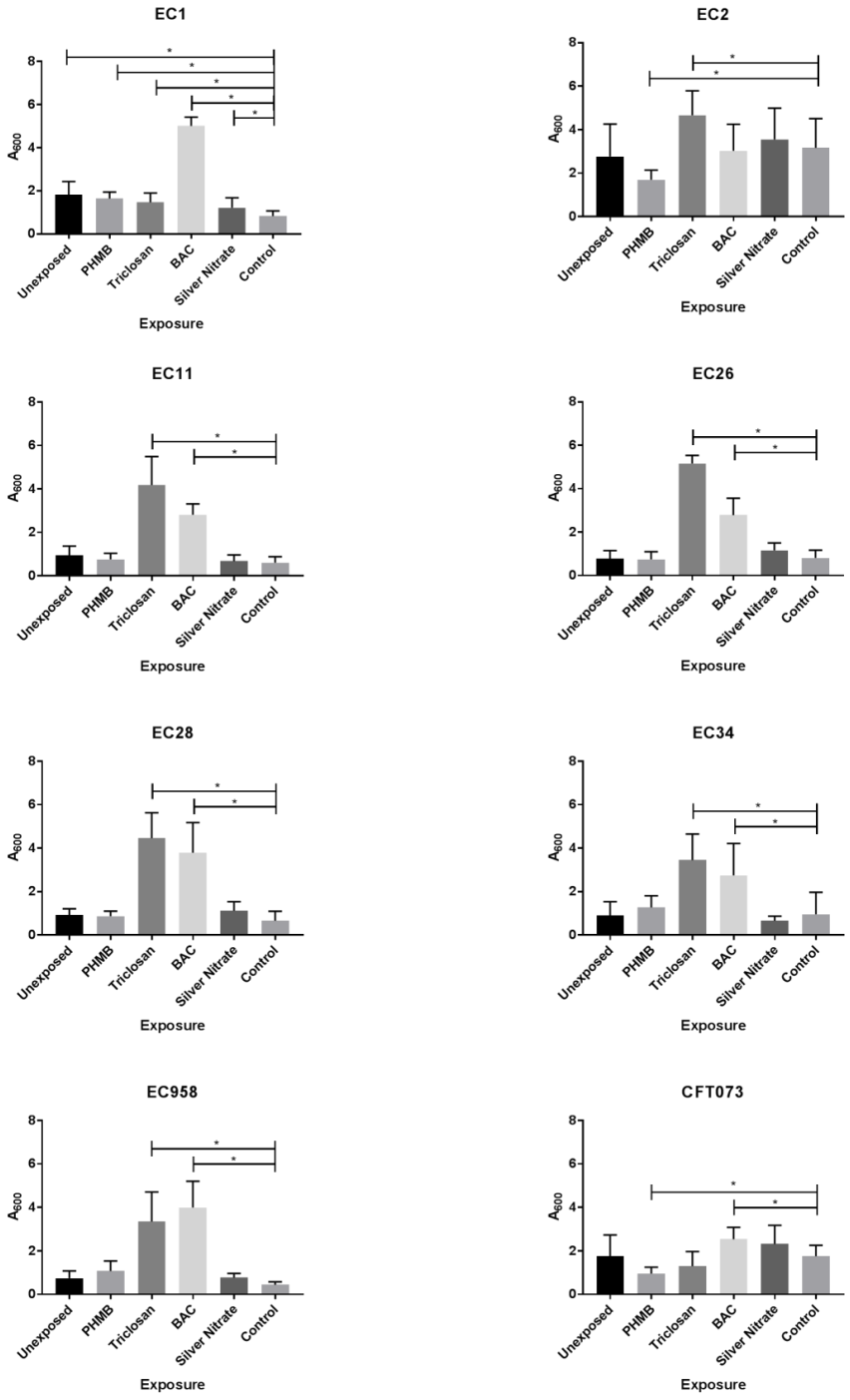

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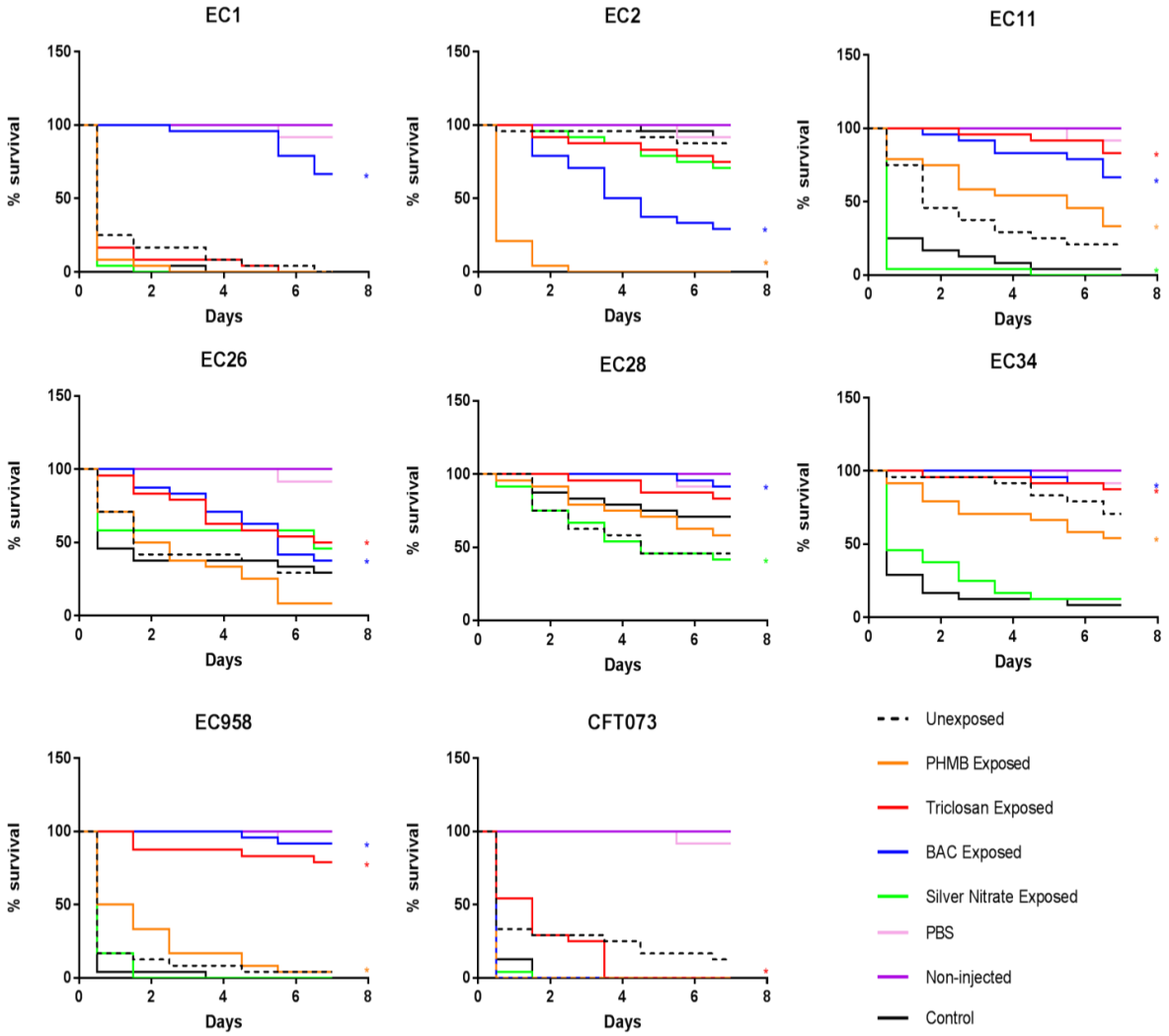
Figure 1: Crystal violet biofilm assay indicating the effect of previous biocide exposure on biofilm formation in eight isolates of UPEC. Data shows the mean absorbance (A_{600}) representative of biofilm formation for individual bacteria before and after long-term exposure to PHMB, triclosan, BAC or silver nitrate or after passage on a biocide free media (Control). Data represent samples taken from two separate experiments each with four technical replicates. For data that varied between replicates, SDs are given as error bars. Significance was determined using ANOVA; * $p \leq 0.05$.

676 Figure 2: Relative pathogenicity of biocide adapted UPEC



677 Figure 2: *G. mellonella* survival curves for larvae injected with unexposed and biocide-
678 exposed UPEC. Data represents 24 biological replicates. Data from non-injected larvae,
679 larvae injected with PBS alone, and larvae injected with control isolates passaged on a
680 biocide free media (Control; C12) are also shown. * indicates a significant difference in
681 pathogenicity when comparing biocide adapted isolates to the respective control strain
682 ($p \leq 0.05$, log-rank reduction test).
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686 Table 1: Minimum inhibitory concentrations for UPEC before and after biocide exposure.

	PHMB			Triclosan			BAC			Silver Nitrate		
Isolate	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**
EC1	0.5	0.2	0.5	0.00001	2	0.02 (0.01)	15.6	15.6	15.6	31.3	62.5	31.3
EC2	0.2	0.2	0.2	0.1	15.6	0.05 (0.02)	15.6	31.3	15.6	31.3	62.5	31.3
EC11	0.2	0.2	0.2	0.1	2	0.05 (0.02)	15.6	31.3	15.6	31.3	62.5	31.3
EC26	0.5	0.2	0.5	0.2	125	0.03	15.6	31.3	15.6	31.3	62.5	31.3
EC28	0.5	0.5	0.5	0.2	3.9	0.2 (0.06)	15.6	15.6	15.6	31.3	62.5	31.3
EC34	0.2	0.2	0.2	0.03	15.6	0.02	15.6	15.6	15.6	31.3	62.5	31.3
EC958	1	0.2	1	0.1	7.8	0.03	15.6	31.3	15.6	31.3	62.5	31.3
CFT073	1	0.2	1	0.1	15.6	0.02	15.6	15.6	15.6	31.3	31.3	15.6

687 Minimum inhibitory concentrations (μg/ml) for UPEC before exposure to biocide (P0), after
688 12 passages in the presence of each biocide (P12), and after 12 passages in a biocide free
689 environment (C12). Data represent mean MICs taken from two separate experiments each
690 with four technical replicates.

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691 Table 2: Minimum bactericidal concentrations for UPEC before and after biocide exposure.

	PHMB			Triclosan			BAC			Silver Nitrate		
Isolate	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**
EC1	1	0.5	0.7 (0.3)	0.002	7.8	7.8	15.6	31.3	15.6	31.3	62.5	31.3
EC2	1	0.5	1	7.8	31.3	7.8	31.3	31.3	15.6	31.3	62.5	31.3
EC11	1	0.5	0.5	7.8	7.8	7.8	15.6	31.3	15.6	31.3	62.5	31.3
EC26	0.5	0.5	0.5	7.8	125	7.8	62.5	31.3	15.6	31.3	62.5	31.3
EC28	1	1	1	7.8	7.8	7.8	31.3	15.6	19.5 (8)	31.3	62.5	31.3
EC34	1	0.5	0.7 (0.3)	7.8	62.5	7.8	15.6	15.6	15.6	31.3	62.5	31.3
EC958	2	1	1.1 (0.5)	7.8	62.5	7.8	62.5	15.6	15.6	31.3	500	31.3
CFT073	15.6	1	1.1 (0.5)	7.8	31.3	7.8	15.6	15.6	15.6	31.3	31.3	15.6

692 Minimum bactericidal concentrations (μg/ml) for UPEC before exposure to biocide (P0),
693 after 12 passages in the presence of each biocide (P12), and after 12 passages in a biocide
694 free environment (C12). Data represent mean MBCs taken from two separate experiments
695 each with four technical replicates.

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700 Table 3: Minimum biofilm eradication concentrations for UPEC before and after biocide
701 exposure.

	PHMB			Triclosan			BAC			Silver Nitrate		
Isolate	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**	**P0**	**P12**	**C12**
EC1	31.3	2000	93.8 (36)	7.8	31.3	0.5	250	500	125	2000	3000	3000
EC2	31.3	2000	93.8 (36)	3.9	250	2	125	500	62.5	3000	3000	3000
EC11	31.3	250	7.8	2	125	0.06	125	125	13.7 (4)	3000	3000	54.7 (16)
EC26	31.3	500	78.1 (31)	1	5000	2	250	250	93.8 (36)	2500	3000	3000
EC28	62.5	2000	62.5	3.9	125	7.8	125	125	125	4000	3000	2750 (500)
EC34	15.6	500	7.8	1	250	0.2 (0.07)	62.5	62.5	11.7 (5)	3000	3000	1750 (975)
EC958	62.5	1000	23.5 (9)	7.8	125	1	250	250	62.5	4000	4000	3000
CFT073	31.3	500	35.2 (20)	2	500	1	62.5	250	62.5	2000	500	1500 (577)

702 Minimum biofilm eradication concentrations (µg/ml) for UPEC before exposure to biocide
703 (P0), after 12 passages in the presence of each biocide (P12), and after 12 passages in a
704 biocide free environment (C12). Data represent mean MBECs taken from two separate
705 experiments each with four technical replicates.

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

Antibiotic	Exposure	EC1	EC2	EC11	EC26	EC28	EC34	EC958	CFT073
Trimethoprim Sulfamethoxazole	Unexposed	31.8 (1.3) S	0 **R**	0 **R**	0 **R**	0 **R**	0 **R**	0 **R**	30 (0.6) S
	PHMB	31.7 (0.8) S	30.3 (0.6) S	0 **R**	0 **R**	0 **R**	0 **R**	0 **R**	0 **R**
	Triclosan	31.5 (0.6) S	0 **R**	32.3 (0.3) S	0 **R**	0 **R**	0 **R**	0 **R**	28.8 (0.4) S
	BAC	29.7 (3.6) S	26 S	31 (0.6) S	0 **R**	0 **R**	0 **R**	0 **R**	28.8 (1.3) S
	Silver Nitrate	32.7 (0.6) S	25.5 (0.5) S	0 **R**	0 **R**	0 **R**	0 **R**	0 **R**	29.5 (0.5) S
Nitrofurantoin	Unexposed	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
	PHMB	20.3 (0.9) S	19.5 (0.5) S	25 (1) S	23.2 (3.1) S	19.2 (0.4) S	15 (0.6) S	19.8 (0.25) S	17.8 (0.8) S
	Triclosan	20 (0.6) S	20.5 (0.6) S	24.7 (0.3) S	24.2 (1.5) S	18.7 (1.4) S	18 (0.6) S	0 **R**	21.2 (0.4) S
	BAC	19.3 (0.3) S	18.5 (0.5) S	23.8 (0.3) S	23.3 (3.1) S	19.5 (1.5) S	15.2 (1.2) S	20.4 (0.1) S	17.2 (0.4) S
	Silver Nitrate	20.3 (0.3) S	18.8 (0.3) S	23.5 (1.6) S	23.3 (2.1) S	21.3 (0.5) S	15.8 (0.8) S	20 S	17.2 (0.4) S
Ciprofloxacin	Unexposed	31.2 (0.8) S	34 (0.6) S	13.8 (0.6) **R**	0 **R**	30 S	0 **R**	0 **R**	0 **R**
	PHMB	31.3 (0.8) S	35 (0.6) S	0 **R**	0 **R**	30 S	0 **R**	0 **R**	0 **R**
	Triclosan	32.5 (0.5) S	0 **R**	29.5 (0.8) S	0 **R**	30.7 (1) S	0 **R**	0 **R**	33.2 (1.9) S
	BAC	30 (0.3) S	0 **R**	0 **R**	0 **R**	29.7 (0.8) S	0 **R**	0 **R**	31.2 (1.8) S
	Silver Nitrate	31.2 (0.3) S	0 **R**	0 **R**	0 **R**	29.7 (0.8) S	0 **R**	0 **R**	31.7 (2.1) S
Gentamicin	Unexposed	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
	PHMB	25.5 (0.6) S	28.1 (0.4) S	27.3 (0.3) S	11.8 (0.8) **R**	18.5 (1.6) S	16.2 (1.2) **I**	26.4 (0.5) S	25 S
	Triclosan	25.8 (0.3) S	16 **I**	23.5 (0.6) S	15.8 (3.5) **I**	20.8 (1) S	20 (0.9) S	27 (0.6) S	28.3 (1.4) S
	BAC	26.5 (0.5) S	25.8 (0.8) S	25.3 (0.3) S	16.8 (0.8) **I**	18.8 (0.8) S	18 S	26.7 (0.6) S	24.7 (0.5) S
	Silver Nitrate	27.8 (0.3) S	27.2 (0.1) S	27 (0.3) S	15.2 (1.5) **I**	18.7 (0.8) S	17 (1.3) S	26.2 (0.3) S	24 S

706 Data show the mean antibiotic inhibition zones (mm) for UPEC before and after biocide exposure (mm) and represent samples taken from two
707 separate experiments each with three technical replicates. For data that varied between replicates, SDs are given in parentheses. S = Sensitive, **I**
708 = Intermediate, **R** = Resistant, as defined by BSAC breakpoint (23).

709 Table 5: Biocide cytotoxicity in an L929 murine fibroblast cell line

Biocide	NR IC_{50}	MTT IC_{50}	m.w.	Mean IC_{50}	
				mg/ ml	mmol/ ml
PHMB	0.02	0.03	2800	0.026	0.000009
Triclosan	0.19	0.14	289.54	0.16	0.00057
BAC	0.07	0.03	340	0.047	0.00014
Silver Nitrate	0.002	0.003	169.87	0.0027	0.000016

710 Mean concentration of biocides allowing 50% survival (IC_{50}) of murine fibroblasts after 30
711 min at 37ºC as determined via NR and MTT assays. Mean IC_{50} based on mass and molecular
712 weight (m.w.). Data indicates two separate experiments each with six replicates.

713 Table 6: Concentration of biocide producing 3 $\log_{10}$ reduction (rf) on eight isolates of UPEC determined by quantitative suspension test and the
714 resulting BI

Biocide	EC1		EC2		EC11		EC26		EC28		EC34		EC958		CFT073	
	rf	BI	rf	BI	rf	BI	rf	BI	rf	BI	rf	BI	rf	BI	rf	BI
PHMB	0.02	1.6	0.06	0.4	0.01	1.6	0.02	1.6	0.3	0.1	0.1	0.2	0.5	0.05	0.02	1.6
Triclosan	0.2	0.7	1.1	0.1	0.2	0.7	1.1	0.1	NC	NC	0.6	0.3	2.3	0.07	NC	NC
BAC	0.07	0.7	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.3	0.2	0.2	0.6	0.08	0.07	0.7
Silver Nitrate	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	0.01	0.2

715 Data shows the concentration of biocide (mg/l) producing 3 $\log_{10}$ reduction (rf) after 30 min of exposure at 37ºC on eight isolates of UPEC and
716 the resulting BI value. NC- not calculable, for certain combination of biocide and bacterial isolate the rf value exceeded the maximum solubility
717 of the biocide. Data represent mean rf values taken from two separate experiments each with four technical replicates.

718 Table 7: Mutation rate frequencies for unexposed UPEC

Isolate	Mutation Rate
EC1	8 (1.7) x 10^{-8}
EC2	3 (1) x 10^{-7}
EC11	1.4 (0.4) x 10^{-7}
EC26	3.4 (0.3) x 10^{-8}
EC28	1.9 x (1) x 10^{-7}
EC34	6.8 (5.4) x 10^{-8}
EC958	3.5 (2.3) x 10^{-8}
CFT073	1.7 (1) x 10^{-8}

719

720 Mutation rate frequencies in UPEC isolates resulting in rifampicin resistance, standard deviations are shown in the parenthesis (n=3).