

## Loss of Function in Escherichia coli exposed to Environmentally Relevant Concentrations of Benzalkonium Chloride

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### Published version

FORBES, Sarah, MORGAN, Nicola, HUMPHREYS, Gavin J., AMÉZQUITA, Alejandro, MISTRY, Hitesh and MCBAIN, Andrew J. (2018). Loss of Function in Escherichia coli exposed to Environmentally Relevant Concentrations of Benzalkonium Chloride. Applied and environmental microbiology.

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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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20 **Abstract**

21 **Background:** Uropathogenic *Escherichia coli* (UPEC) are a frequent cause of catheter  
22 associated urinary tract infection (CAUTI). Biocides have been incorporated into catheter-  
23 coatings to inhibit bacterial colonisation whilst ideally exhibiting low cytotoxicity and  
24 mitigating the selection of resistant bacterial populations. We compared the effects of long-  
25 term biocide exposure on susceptibility, biofilm-formation and relative-pathogenicity in eight  
26 UPEC isolates. **Methods:** Minimum inhibitory concentrations (MIC), minimum bactericidal  
27 concentrations (MBC), minimum biofilm eradication concentrations (MBEC) and antibiotic  
28 susceptibilities were determined before and after long-term exposure to triclosan,  
29 polyhexamethylene biguanide (PHMB), benzalkonium chloride (BAC) and silver nitrate.  
30 Biofilm-formation was quantified using a crystal violet assay and relative-pathogenicity was  
31 assessed via a *Galleria mellonella* waxworm model. Cytotoxicity and resulting  
32 biocompatibility index values were determined against an L929 murine fibroblast cell line.  
33 **Results:** Biocide exposure resulted in multiple decreases in biocide susceptibility in  
34 planktonic and biofilm associated UPEC. Triclosan exposure induced the largest frequency  
35 and magnitude of susceptibility decreases at MIC, MBC and MBEC, which correlated to an  
36 increase in biofilm biomass in all isolates. Induction of antibiotic-cross-resistance occurred in  
37 6/84 possible combinations of bacteria, biocide and antibiotic. Relative-pathogenicity  
38 significantly decreased after triclosan exposure (5/8 isolates), increased after silver nitrate  
39 exposure (2/8 isolates) and varied between isolates for PHMB and BAC. Biocompatibility  
40 index ranked antiseptic potential as PHMB>triclosan>BAC>silver nitrate. **Conclusion:**  
41 Biocide exposure in UPEC may lead to reductions in biocide and antibiotic susceptibility,  
42 changes in biofilm-formation and alterations relative-pathogenicity. These data indicate the  
43 multiple consequences of biocide adaptation that should be considered when selecting an  
44 anti-infective catheter-coating agent.

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46

47 **Introduction**

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

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

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

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

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

## 106 **Methods**

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

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

119 brief, 100  $\mu$ l of a 5  $\times$  MBC concentration solution of biocide was added to an 8 x 8 mm well  
120 in the centre of a 90 mm agar plate. Bacterial pure cultures were radially inoculated in  
121 duplicate from the edge of the plate to the centre, prior to incubation for 2 days aerobically at  
122 37°C. Biomass from the inner edge of the annulus of bacterial growth representative of the  
123 highest biocide concentration at which growth could occur was removed and used to  
124 inoculate a new biocide containing plate, as outlined above. This process was repeated for 12  
125 passages. Control isolates passaged 12 times on biocide free media were also included.  
126 Bacteria were archived at -80 °C before and after biocide passage for subsequent testing.

127 **Minimum inhibitory and minimum bactericidal concentration.** Minimum  
128 inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were  
129 determined as described previously (17). In brief, 2  $\times$  5 ml overnight cultures of test bacteria  
130 were prepared in MHB prior to overnight incubation (18-24 h) at 37°C and 100 rpm. Cultures  
131 were diluted to an OD<sub>600</sub> of 0.008 in 20 ml of sterile MHB to produce a bacterial inoculum for  
132 biocide susceptibility testing. Doubling dilutions (150  $\mu$ l) of each test biocide were prepared  
133 in sterile MHB in a 96-well microtiter plate prior to addition of bacterial inoculum (150  $\mu$ l).  
134 Plates were incubated overnight (18-24 h) at 37°C and 100 rpm. The MIC was defined as the  
135 lowest concentration of biocide for which growth was completely inhibited (viewed as  
136 turbidity relative to a sterile negative control). To determine MBC aliquots (5  $\mu$ l) were taken  
137 from the wells of the MIC plate and were spot plated onto Muller Hinton Agar (MHA) in  
138 triplicate. The plates were incubated statically for 18-24 h at 37°C. The lowest test  
139 concentration for which visible bacterial growth was completely inhibited was deemed the  
140 MBC.

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

144 and were incubated for 18-24 h at 37°C and 100 rpm before being diluted to an OD<sub>600</sub> of  
145 0.008 in MHB to create a bacterial inoculum for biofilm susceptibility testing. 100 µl of  
146 bacterial inoculum was added to each well of the CBD base, plates were incubated at 37°C  
147 for 48 h to allow biofilm formation on the pegs. Doubling dilutions of biocides were prepared  
148 in sterile broth across a 96-well microtiter plate. Biofilms were exposed to antimicrobial  
149 compounds and incubated for 24 h at 37°C and 100 rpm. After incubation, the pegged lid was  
150 transferred to a 96-well plate containing 200 µl of sterile broth and was incubated for 24 h at  
151 37°C and 100 rpm. MBEC was defined as the lowest concentration of biocide for which re-  
152 growth was completely inhibited (viewed as turbidity relative to a sterile negative control)  
153 indicating complete biofilm eradication.

154 **Crystal violet bacterial attachment assay.** 2 × 5 ml overnight cultures of test  
155 bacteria were diluted to an OD<sub>600</sub> of 0.008 in MHB after incubation for 18-24h at 37° C and  
156 100 rpm. 150 µl of diluted overnight bacterial culture was added to the wells of a sterile 96-  
157 well microtiter plate. Plates were incubated statically for 48 h at 37°C. Media was removed  
158 from wells and replaced with 180 µl of crystal violet solution. The plate was left at room  
159 temperature for 30 minutes, crystal violet solution was decanted and the wells were rinsed  
160 with 3 × 200 µl of PBS prior to drying for 1 h at 37°C. The remaining crystal violet was  
161 solubilised in 250 µl of 100% ethanol. The A<sub>600</sub> of the solubilised crystal violet solution was  
162 determined and compared to a sterile MHB negative control.

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



169 counts on MHA). Aliquots of each suspension (5  $\mu$ l) 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  $\mu$ l  
188 aliquots of inoculum were then transferred into 135  $\mu$ l of biocide containing cell culture  
189 medium prior to incubation for 30 min at 37°C. For PHMB, BAC, and triclosan, the biocide  
190 was subsequently inactivated by transfer of 15  $\mu$ l of the suspension into 135  $\mu$ L 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,

194 5  $\mu$ l aliquots were spot plated onto MHA in triplicate. The plates were incubated statically for  
195 18-24 h at 37°C and CFU/ml was determined. The lowest test concentration which achieved  
196 at least a  $3\log_{10}$  (99.9%) reduction in bacterial load was deemed the rf value. BI is calculated  
197 as  $IC_{50}/rf$  for each combination of biocide and isolate and indicates the antiseptic potential of  
198 the test compound.

199 **Antibiotic susceptibility.** Bacterial susceptibility was determined for trimethoprim  
200 sulfamethoxazole (25  $\mu$ g), nitrofurantoin (50  $\mu$ g), ciprofloxacin (10  $\mu$ g), and gentamicin (200  
201  $\mu$ g). Antibiotic susceptibility tests were performed according to the standardized British  
202 Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method for antimicrobial  
203 susceptibility testing (23).

204 **Determination of mutation rate frequency.** Mutation rate frequency was  
205 determined as described by Miller *et al* (24) In brief, 100  $\mu$ l aliquots of diluted overnight  
206 culture obtained from single bacterial colonies were plated onto antibiotic free MHA plates  
207 and MHA plates containing 50 $\mu$ g/ml rifampicin in triplicate. Plates were incubated for 24h at  
208 37°C prior to determination of viable count. Mutation frequencies were expressed as the  
209 number of resistant mutants recovered as a fraction of total viable bacteria.

## 210 **Results**

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

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

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

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

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

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

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

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

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

290 **Discussion**

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

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

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

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

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

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

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

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

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



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

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

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

384 BAC adaptation has been previously correlated to an increase in biofilm biomass in *E. coli*  
385 which is believed to be due to an increase in protein and polysaccharide content within the  
386 extracellular polymeric substance (EPS) (41). This change in EPS composition may lead to  
387 reduced BAC susceptibility, as observed in our BAC adapted isolates. Yu *et al.* (42) utilised a  
388 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

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

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

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

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

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

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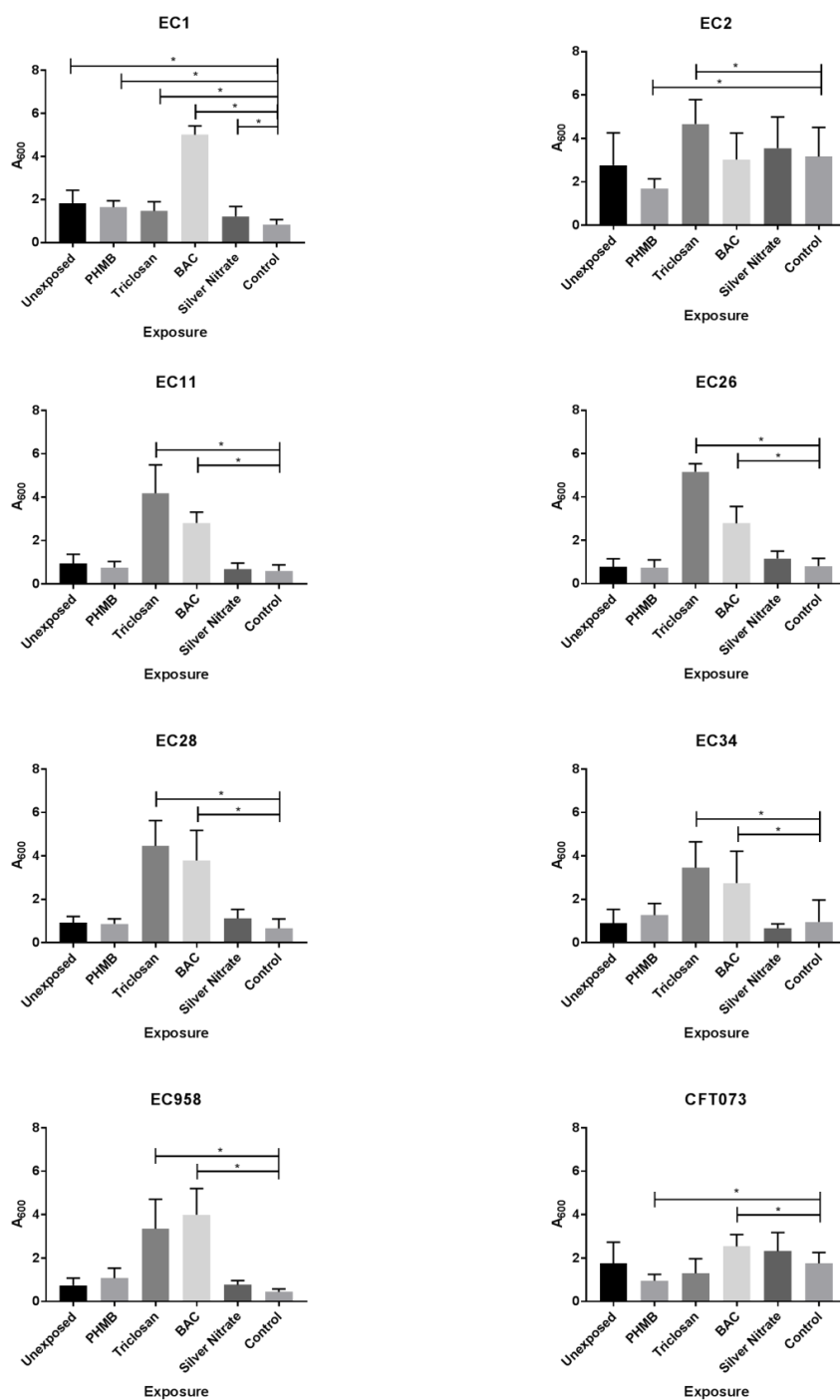
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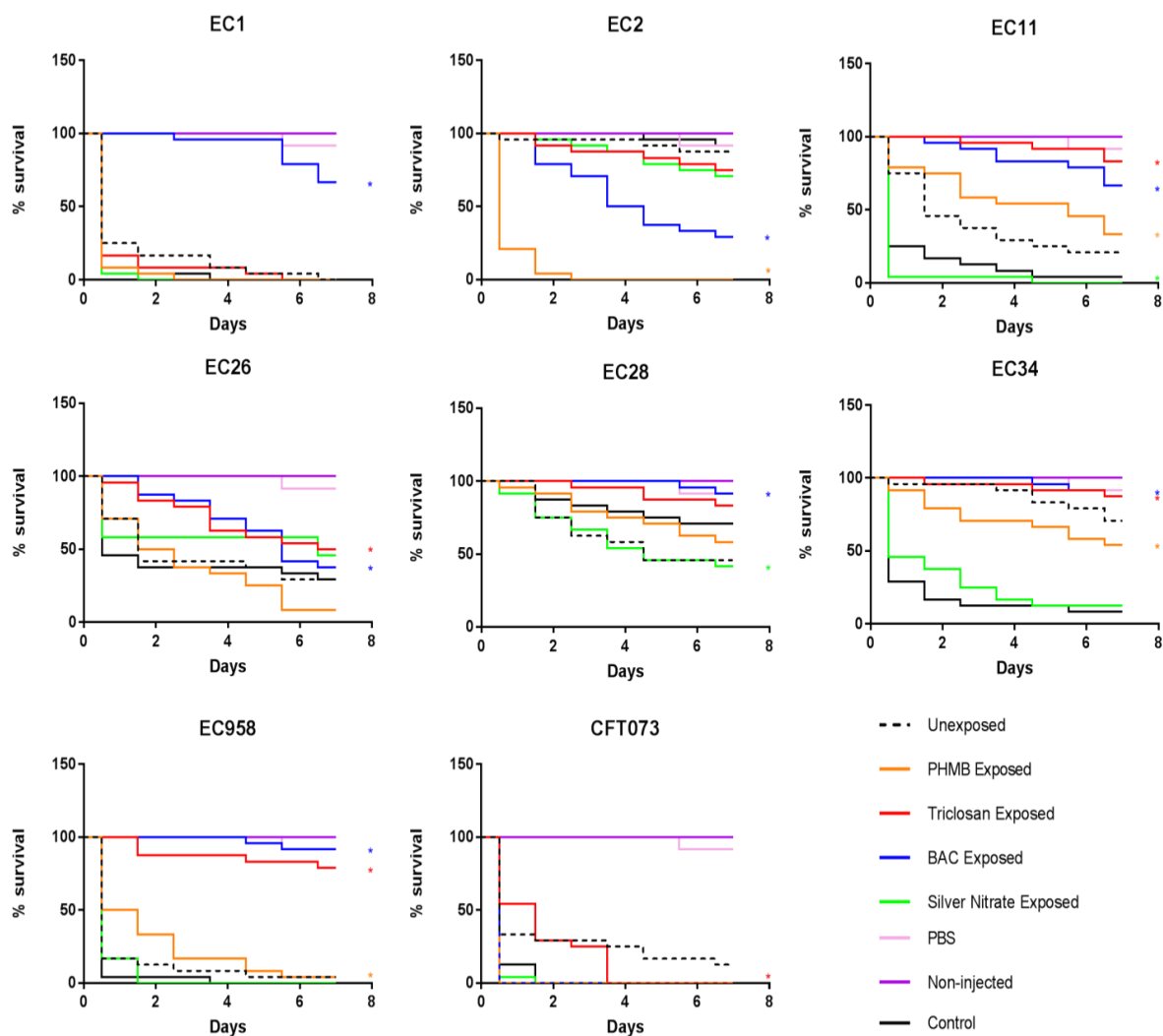
667 Figure 1: Biofilm formation in biocide adapted UPEC



668

669 Figure 1: Crystal violet biofilm assay indicating the effect of previous biocide exposure on  
670 biofilm formation in eight isolates of UPEC. Data shows the mean absorbance ( $A_{600}$ )  
671 representative of biofilm formation for individual bacteria before and after long-term  
672 exposure to PHMB, triclosan, BAC or silver nitrate or after passage on a biocide free media  
673 (Control). Data represent samples taken from two separate experiments each with four  
674 technical replicates. For data that varied between replicates, SDs are given as error bars.  
675 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).

683

684

685



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

Isolate	PHMB			Triclosan			BAC			Silver Nitrate		
	P0	P12	C12	P0	P12	C12	P0	P12	C12	P0	P12	C12
<b>EC1</b>	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
<b>EC2</b>	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
<b>EC11</b>	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
<b>EC26</b>	0.5	0.2	0.5	0.2	125	0.03	15.6	31.3	15.6	31.3	62.5	31.3
<b>EC28</b>	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
<b>EC34</b>	0.2	0.2	0.2	0.03	15.6	0.02	15.6	15.6	15.6	31.3	62.5	31.3
<b>EC958</b>	1	0.2	1	0.1	7.8	0.03	15.6	31.3	15.6	31.3	62.5	31.3
<b>CFT073</b>	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 ( $\mu\text{g/ml}$ ) 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.

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

Isolate	PHMB			Triclosan			BAC			Silver Nitrate		
	P0	P12	C12	P0	P12	C12	P0	P12	C12	P0	P12	C12
<b>EC1</b>	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
<b>EC2</b>	1	0.5	1	7.8	31.3	7.8	31.3	31.3	15.6	31.3	62.5	31.3
<b>EC11</b>	1	0.5	0.5	7.8	7.8	7.8	15.6	31.3	15.6	31.3	62.5	31.3
<b>EC26</b>	0.5	0.5	0.5	7.8	125	7.8	62.5	31.3	15.6	31.3	62.5	31.3
<b>EC28</b>	1	1	1	7.8	7.8	7.8	31.3	15.6	19.5 (8)	31.3	62.5	31.3
<b>EC34</b>	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
<b>EC958</b>	2	1	1.1 (0.5)	7.8	62.5	7.8	62.5	15.6	15.6	31.3	500	31.3
<b>CFT073</b>	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 ( $\mu\text{g/ml}$ ) 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.

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

702 Minimum biofilm eradication concentrations ( $\mu\text{g/ml}$ ) 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 <sub>50</sub>	MTT IC <sub>50</sub>	m.w.	Mean IC <sub>50</sub>	
				mg/ ml	mmol/ ml
<b>PHMB</b>	0.02	0.03	2800	0.026	0.000009
<b>Triclosan</b>	0.19	0.14	289.54	0.16	0.00057
<b>BAC</b>	0.07	0.03	340	0.047	0.00014
<b>Silver Nitrate</b>	0.002	0.003	169.87	0.0027	0.000016

710 Mean concentration of biocides allowing 50% survival (IC<sub>50</sub>) of murine fibroblasts after 30  
711 min at 37°C as determined via NR and MTT assays. Mean IC<sub>50</sub> 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<sub>10</sub> 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
<b>PHMB</b>	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
<b>Triclosan</b>	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
<b>BAC</b>	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
<b>Silver Nitrate</b>	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<sub>10</sub> reduction (rf) after 30 min of exposure at 37°C 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	$8 (1.7) \times 10^{-8}$
EC2	$3 (1) \times 10^{-7}$
EC11	$1.4 (0.4) \times 10^{-7}$
EC26	$3.4 (0.3) \times 10^{-8}$
EC28	$1.9 \times (1) \times 10^{-7}$
EC34	$6.8 (5.4) \times 10^{-8}$
EC958	$3.5 (2.3) \times 10^{-8}$
CFT073	$1.7 (1) \times 10^{-8}$

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