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Variable Effects of Exposure to Formulated Microbicides on Antibiotic Susceptibility in Firmicutes and Proteobacteria

Sarah Forbes[1], Christopher G Knight[2], Nicola L Cowley[1], Alejandro Amézquita[3], Peter McClure[3], Gavin Humphreys[1] and Andrew J McBain[1*]

[1]Manchester Pharmacy School and [2]Faculty of Life Sciences, The University of Manchester, Manchester, UK.

[3]Unilever Safety and Environmental Assurance Centre, Colworth Science Park, Bedford UK.

Key words Microbicides, biocides, antibiotics, susceptibility, resistance, formulation.

Running title: Antibiotic susceptibility following exposure to microbicides

*For correspondence: Andrew McBain, Manchester Pharmacy School, The University of Manchester, Oxford Road, Manchester M13 9PT, UK. Tel: 44 161 275 2360; Fax: 44(0)161 275 2396; Email: andrew.mcbain@manchester.ac.uk

ABSTRACT

Microbicides are broad-spectrum antimicrobial agents that generally interact with multiple pharmacological targets. Whilst they are widely deployed in disinfectant, antiseptic and preservative formulations, data relating to their potential to select for microbicide or antibiotic resistance have been generated mainly by testing the compounds in much simpler aqueous solutions. In the current investigation, antibiotic susceptibility was determined for bacteria that had previously exhibited decreased microbicide susceptibility following repeated exposure to microbicides either in formulation with sequestrants and surfactants or in simple aqueous solution. Statistically significant increases in antibiotic susceptibility occurred for 12% of bacteria after exposure to microbicides in formulation *vs* 20% after exposure to aqueous solutions, whilst 22% became significantly less susceptible to the antibiotics, regardless of formulation. Of the combinations of bacterium and antibiotic for which British Society for Antimicrobial Chemotherapy breakpoints are available, none became resistant. Linear modeling, taking into account phylogeny, microbicide, antibiotic and formulation identified small but significant effects of formulation that varied depending on bacterium and microbicide. Adaptation to formulated benzalkonium chloride in particular was more likely to increase antibiotic susceptibility than the simple aqueous solution. In conclusion, bacterial adaptation through repeated microbicide-exposure was associated with both increases and decreases in antibiotic susceptibility. Formulation of the microbicide to which the bacteria had previously adapted had an identifiable effect on antibiotic susceptibility but this was typically small relative to the differences observed among microbicides. Susceptibility changes resulting in resistance were not observed.

IMPORTANCE

The safety of certain microbicide applications has been questioned due to the possibility microbicide exposure could select for microbicide and antibiotic resistance. Evidence that this may happen is based mainly on in vitro experiments where bacteria have been exposed to microbicides in aqueous solution. Microbicides are however normally deployed in products formulated with surfactants, sequestrants and other compounds. Whilst this may influence the frequency and extent of susceptibility changes, few reports in the literature have assessed this. In the current investigation therefore we have investigated changes in antibiotic susceptibility in bacteria, which exhibited decreased microbicide susceptibility following repeated exposure to microbicides in simple aqueous solutions and in formulation. We report that microbicide formulation had an identifiable effect on antibiotic susceptibility but this was typically small relative to the differences observed among microbicides. Susceptibility changes resulting in resistance were not observed.

INTRODUCTION

Microbicides are broad-spectrum antimicrobial compounds that are widely deployed to control the growth of microorganisms or eliminate them. Applications include the control of biofouling and microbial contamination in industry (1) as well as clinical antisepsis (2-4). They are also used extensively in the domestic environment as hygiene adjuncts and preservatives in a range of formulations including oral care products (5), hand sanitizers (6) and hard surface cleaners (7).

The safety of certain microbicide applications has been questioned due to the possibility that long-term microbicide exposure could select for reduced antimicrobial susceptibility in bacteria (8-10). Reduced microbicide susceptibility has been reported for some combinations of bacterium and microbicide (11) and changes in bacterial susceptibility to chemically unrelated antimicrobials such as antibiotics or other microbicides have been reported following laboratory microbicide exposure (12, 13). The mechanisms involved in such cross-resistance include selection for mutations in shared cellular target sites, upregulation of efflux pumps (14), reductions in cell permeability (15) and in some cases, sporulation (16).

Evidence that microbicides can select for reduced microbicide susceptibility in the environment is limited, with the majority of reports relating to *in vitro* exposure (17). Similarly, little evidence has emerged to firmly link microbicide/antibiotic cross-resistance to microbicide use (18-21). The majority of studies aiming to better understand the potential risks of resistance through microbicide exposure have exposed bacteria to microbicides in aqueous solution with or without the addition of co-solvents such as dimethyl sulfoxide (22) or ethanol (23). In real use however, microbicides are deployed in products formulated with surfactants, sequestrants and other compounds that can interact with cellular targets to influence antimicrobial potency. As previously reported, such formulation can decrease the frequency and extent of the acquisition of reduced microbicide susceptibility in bacteria (24).

Accordingly, evaluating the effects of bacterial exposure to microbicides within a formulation chassis containing surfactants and sequestrants may generate more realistic data on which to base risk assessments on the induction of changes in bacterial susceptibility. In the current investigation we have therefore assessed changes in antibiotic susceptibility in bacteria which have previously exhibited decreases in microbicide susceptibility following repeated exposure to a range of microbicides in simple aqueous solutions and in formulations containing commonly used non-ionic surfactants and sequestrants (24). The microbicides tested reflect those frequently used in consumer products such as laundry detergents, hard surface disinfectants and personal care products. The antibiotics were selected on the basis of their common therapeutic use and their inclusion in a US investigation of links between domestic microbicide use and antibiotic resistance (25).

MATERIALS AND METHODS

Bacteria. *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, and *Escherichia coli* ATCC 25922 were obtained from Oxoid (Basingstoke, United Kingdom). *Acinetobacter baumannii* MBRG15.1, *Pseudomonas putida* MBRG15.2, *Escherichia coli* MBRG15.4 and *Cronobacter sakazakii* MBRG15.5, were isolated from a domestic kitchen drain biofilm. *Enterococcus faecalis* MRBG15.6 is a wound isolate provided by Angela Oates, The University of Manchester.

Chemicals reagents and growth media. Bacteriological growth media were purchased from Oxoid (Basingstoke, United Kingdom). All other chemical reagents were purchased from Sigma-Aldrich (Dorset, United Kingdom) unless otherwise stated. Bacterial growth media were sterilized at 121°C and 15 lb/in^2 for 15 min prior to use. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus faecalis* were cultured on Tryptone Soy agar and broth. *Acinetobacter baumannii*, *Pseudomonas putida* and

119 *Cronobacter sakazakii* were grown on Wilkins Chalgren agar and broth containing 2%
120 sucrose. All bacteria were incubated aerobically at 37°C for 18 h unless stated otherwise.

121 **Antimicrobials.** The microbicides benzalkonium chloride (BAC), chlorhexidine
122 digluconate (CHX 20% v/v), thymol and triclosan were purchased from Sigma-Aldrich
123 (Dorset, UK). Didecyldimethyl ammonium chloride (DDAC 50% v/v) was purchased from
124 Merck Millipore (Durham, UK). Vantocil (a 20% v/v aqueous solution of polyhexamethylene
125 biguanide (PHMB) was obtained from Arch Chemicals Inc. (Manchester, UK). Glydant (1,3-
126 Dimethylol-5,5-dimethylhydantoin; DMDM hydantoin at 54% v/v) was obtained from Lonza
127 (Bishops Stortford, UK) whilst benzisothiazolinone (BIT) was supplied by Unilever (Port
128 Sunlight, UK). All microbicides were prepared in aqueous solution or added to a microbicide-
129 free formulation chassis containing sequestrants and surfactants as previously described (24),
130 at concentrations reflective of their normal deployment in consumer products. BAC, CHX,
131 DDAC, DMDM hydantoin, PHMB and thymol were prepared at 1% (v/v) in a general
132 purpose cleaner. Triclosan was added to a laundry detergent at 0.0066% (w/v).
133 Benzisothiazolinone was formulated into a laundry detergent at 0.02% (v/v). Ampicillin
134 (10µg), cephalothin (20µg), ciprofloxacin (1µg), kanamycin (5µg) and tetracycline (10µg)
135 antibiotic discs were obtained from Oxoid (Basingstoke, UK).

136 **16S rRNA gene sequencing**. Single bacterial colonies were dispersed in 100µl of
137 nanopure water, vortexed for 30 sec. and boiled at 100˚C for 15min. to lyse cells.
138 Microcentrifuge tubes were centrifuged at 16, 000 x g for 1 min to remove cellular debris and
139 the resulting supernatant was retained as DNA template. PCR was performed using the
140 primers 8FLP (5'-GAG TTT GAT CCT GGS TCA G-3') and 806R (5'-GGA CTA CCA
141 GGG TAT CTA AT-3') at 5µM per reaction. PCR was conducted using a Biometra
142 TGradient thermocycler (Analytik Jena, Germany) and run for 35 thermal cycles: 94˚C (1
143 min), 53˚C (1 min) and 72˚C (1min). A 15 min. elongation step was included in the final

144 cycle. PCR products were purified using a QIAquick PCR purification kit (Qiagen, West
145 Sussex, UK) according to manufacturer's instructions and the resulting DNA yield was
146 quantified using a NanoDrop 2000c UV-vis spectrophotometer (Thermo Scientific,
147 Wilmington, USA). A reaction mixture containing 4pM forward or reverse primer and 40-
148 50ng of DNA in 10µl total volume was used for DNA sequencing. DNA sequencing was
149 performed using the Applied Biosystems 3730 DNA Analyzer (ThermoFisher, Paisley, UK).

150 **Microbicide exposure in aqueous solution and formulation.** A system previously
151 validated as highly selective for changes in antimicrobial susceptibility (26, 27) was used.
152 Reproducible *c.* 100-fold-concentration gradients of the antimicrobial compounds were
153 generated on Tryptone Soy or Wilkins Chalgren agar plates using an automated spiral plater
154 (Don Whitley Scientific, Shipley, United Kingdom). Antimicrobials in aqueous solution or in
155 formulation (50µl) were deposited on the agar surface. Plates were dried for 1h at room
156 temperature prior to radial deposition of bacterial pure cultures and then incubated (4d; 37°C)
157 in an aerobic incubator. After incubation, growth observed at the highest microbicide
158 concentration was aseptically removed and streaked onto a fresh plate containing the same
159 antimicrobial compound concentration gradient. Where growth was observed across the
160 whole antimicrobial gradient, a new plate produced with a 5-fold-higher microbicide
161 concentration was used. This process was repeated until 14 passages had occurred (P14).
162 Bacteria at P0 and P14 were archived for subsequent susceptibility testing.

163 **Determination of antibiotic susceptibility.** Bacteria showing ≥4-fold increases in
164 minimum bactericidal concentration (MBC) after microbicide/formulation exposure were
165 investigated for changes in antibiotic susceptibility. Antibiotic susceptibilities were
166 determined for ciprofloxacin (1µg), cephalothin (20µg), ampicillin (10µg), kanamycin (5µg)
167 and tetracycline (10µg). Disc diffusion assays were performed according to the British

168 Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method for antimicrobial
169 susceptibility testing (28).

170 **Statistical analyses.** Antibiotic zone of inhibition sizes were compared before and
171 after adaptation to microbicides using Mann-Whitney U tests and in the cross-resistance
172 assays using linear mixed effect models (LMMs). LMMs were required to simultaneously
173 compare and account for the effects on the inhibition zone of: a) microbicidal environment to
174 which the bacterium was adapted, b) the antibiotic against which it was tested and c) the
175 interaction of microbicidal environment and antibiotic (each fitted as fixed effects) plus d) the
176 different bacteria (fitted as a random effect), allowing the variation among bacteria to differ
177 for different antibiotics. Initial models with this structure violated the statistical assumptions
178 of normality of residuals and homogeneity of variance. Box-Cox transformation indicated that
179 a transformation with a power of 0.5 (square root) was approximately optimal to address the
180 non-normality and was therefore used. A wide range of different models accounting for non-
181 homogeneity of variance in response to different variables was tested. Models allowing
182 different variances for different bacteria and different variances for different microbicidal
183 environments were superior to all others tested (lowest Akaike information criterion). To
184 account for the fact that closely related bacteria are likely to respond more similarly than
185 others just through having a more recent common ancestor, a correlation term was included
186 based on the 16S-based phylogenetic tree of the strains used. Testing different weightings on
187 this correlation term (Pagel's λ (29)) determined that a Brownian model (i.e. Pagel's $\lambda = 1$)
188 was best. In addition, a LMM was fitted for the subset of data involving microbicides where
189 bacteria were tested that had adapted to both formulated and unformulated versions of the
190 microbicidal environment. In this case, accounting for non-homogenous variance was best
191 done by allowing different variances for different microbicidal environments and for variance
192 to increase at higher values according to the formula $e^{(0.65 * \text{zone of clearance value})}$. All models were

fitted using the NLME package (Version 3.1) (30) in R version 3.2 (31) with phylogenetic correlation structures created using the APE package (version 3.3) (32). Where p-values are not explicitly given, statistical significance was deemed to be $p< 0{\cdot}05$.

RESULTS

After exposure to microbicides in simple aqueous solution, out of 90 possible combinations of bacterium and antibiotic, 22% significantly ($P < 0.05$) reduced in antibiotic susceptibility (8% towards ciprofloxacin, 6% to ampicillin, 4% to kanamycin, 2% to tetracycline and 2% to cephalothin). In comparison, 20% significantly increased in antibiotic susceptibility (6% towards ciprofloxacin, 4% to kanamycin, 4% to tetracycline, 3% to cephalothin and 2% to ampicillin). After exposure to the formulated microbicides, out of 50 possible combinations of bacterium and antibiotic, 22% significantly decreased in antibiotic susceptibility (6% ciprofloxacin, 6% kanamycin, 4% cephalothin and 4% tetracycline and 2% ampicillin). In comparison, 12% significantly increased in antibiotic susceptibility (8% ciprofloxacin 2% kanamycin and 2% tetracycline). Importantly, whilst statistically significant increases and decreases in antibiotic susceptibility occurred, generation of resistance as defined by BSAC breakpoints was not observed in any previously susceptible bacterium.

The frequency of reduction in antibiotic susceptibility was highest in organisms exhibiting previously reduced susceptibility towards DMDM hydantoin (80%), followed by BAC, CHX, DDAC (20%), triclosan (20%) and PHMB (16%). Bacteria with reduced susceptibility to triclosan showed the highest frequency of increased antibiotic susceptibility (45%), followed by CHX (30%), DDAC (27%), DMDM hydantoin (20%) and PHMB (4%). In comparison, after exposure to the formulations, 27% of thymol formulation and 20% of DDAC formulation-adapted isolates exhibited increased antibiotic susceptibility, whilst 40% of DDAC formulation, 33% of thymol formulation, 10% of BAC formulation and 7% of PHMB

217 formulation-adapted bacteria had significantly decreased antibiotic susceptibility. The
218 following section details the effects of each microbicide on antibiotic susceptibility.

219 **Benzalkonium chloride.** When comparing unexposed to BAC-adapted organisms
220 there was a significant decrease in susceptibility of *S. aureus* to ciprofloxacin and kanamycin
221 (Table 1). *E. coli* also showed a significant reduction in kanamycin susceptibility after
222 exposure to BAC. After repeated exposure to BAC formulation *S. aureus* showed a
223 significantly decreased susceptibility to ciprofloxacin (Table 1).

224 **Chlorhexidine.** *S. aureus* showed a significant decrease in susceptibility to ampicillin
225 and ciprofloxacin after CHX exposure as well as an increase in susceptibility to tetracycline
226 (Table 1). *E. coli* demonstrated increased susceptibility to ciprofloxacin and ampicillin after
227 repeated exposure to chlorhexidine.

228 **Didecydimethyl ammonium chloride.** After exposure to DDAC, *A. baumanii*
229 showed a significant increase in susceptibility to ciprofloxacin and kanamycin and decreased
230 susceptibility to tetracycline when compared to the bacterium before microbicide exposure
231 (Table 1). Increased susceptibility to ciprofloxacin, kanamycin and cephalothin was observed
232 for the *E. coli* drain isolate, whilst a significant reduction in tetracycline susceptibility was
233 also evident in this bacterium. After exposure to DDAC in formulation, the *E. coli* drain
234 isolate underwent a significant reduction in kanamycin, cephalothin, tetracycline and
235 ampicillin susceptibility, and an increase in susceptibility to ciprofloxacin. *P. aeruginosa*
236 showed a significant increase in ciprofloxacin susceptibility after long-term exposure to
237 DDAC formulation (Table 1).

238 **DMDM hydantoin.** After repeated exposure to DMDM hydantoin the *E. coli* drain
239 isolate demonstrated a significant reduction in ciprofloxacin, kanamycin, cephalothin and
240 ampicillin susceptibility and an increase in tetracycline susceptibility when compared to its
241 pre-exposed counterpart (Table 1).

242 **Polyhexamethylene biguanide**. Following adaptation to PHMB, the *E. coli* drain
243 isolate exhibited a decrease in kanamycin and ciprofloxacin susceptibility (Table 1). *S. aureus*
244 developed a significantly reduced susceptibility to ampicillin and ciprofloxacin after repeated
245 PHMB exposure but higher tetracycline susceptibility when compared to the unexposed
246 parent strain. After exposure to PHMB formulation *S. aureus* also showed a significant
247 reduction in ciprofloxacin susceptibility.

248 **Thymol.** None of the test bacteria demonstrated a significant change in antibiotic
249 susceptibility after exposure to thymol in aqueous solution. Following exposure to the
250 thymol-containing formulation however, *P. putida* underwent significant decreases in
251 susceptibility to ciprofloxacin and kanamycin (Table 1), whilst *E. coli* showed significant
252 increases in ciprofloxacin and cephalothin susceptibility but decreases in susceptibility to
253 kanamycin and tetracycline. *A. baumanii* increased in susceptibility to ciprofloxacin,
254 kanamycin and tetracycline compared to its unexposed counterpart (Table 1).

255 **Triclosan**. Following exposure to triclosan, *S. aureus* exhibited significant reductions
256 in ciprofloxacin and ampicillin susceptibility whilst susceptibility to kanamycin, tetracycline
257 and cephalothin increased (Table 1). *E. coli* showed increased susceptibility to ampicillin and
258 ciprofloxacin for this bacterium after triclosan exposure, whilst the *E. coli* drain isolate
259 showed decreased ciprofloxacin susceptibility but increased cephalothin susceptibility, when
260 compared to the parent strain. Comparatively *C. sakazakii* showed a significant increase in
261 ciprofloxacin, cephalothin and kanamycin susceptibility, and a decrease in ampicillin
262 susceptibility after repeated triclosan exposure (Table 1).

263 To gain an overview of the statistical significance of the observed changes in antibiotic
264 susceptibility and ask whether it was possible to identify consistent patterns in susceptibility,
265 linear mixed-effects models were fitted for how the susceptibility to particular antibiotics
266 varied, dependent on the antibiotic in question, the bacterium and the microbicidal

environment previously adapted to. A highly significant interaction ($F_{40, 298} = 15$, $P < 2 \times 10^{-16}$) indicative of different responses to particular antibiotics dependent on the microbicidal environment to which the organism had previously adapted (Fig. 1) was observed. Bacterial strains differed most in their response to ampicillin (standard deviation among strains = 5.1) and least in their response to tetracycline (standard deviation among strains = 2.7), with the responses of different strains to some antibiotics being associated either positively (cephalothin and ampicillin, $r = 0.95$) or negatively (ciprofloxacin and ampicillin, $r = -0.28$), (Table 2).

Data presented in Fig. 1 indicate differences in the antibiotic susceptibility of organisms previously adapted to either formulated or unformulated microbicides. The differences in susceptibility changes observed between microbicides in simple aqueous solution or in complex formulation were highly significant (likelihood ratio test of the full model against a model treating formulated and unformulated versions of microbicides as equivalent: $LR_{88,70} = 61$, $P = 8.6 \times 10^{-10}$). To test whether there was any consistent effect of formulation; a second linear mixed-effects model was created for the subset of the data where strains had adapted to both formulated and unformulated versions of the same microbicide (PHMB, BAC and DDAC). This indicated that the way bacteria adapted to formulated versus non-formulated versions of a microbicide depended on the microbicide in question ($F_{2, 145} = 4.5$, $P = 0.012$), although that did not vary significantly among the antibiotics ($F_{8, 145} = 0.70$, $P = 0.69$). The effect of formulation was specific to BAC, with formulation giving a small increase in the antibiotic susceptibility of microbes adapted to it (Fig. 2).

DISCUSSION

Investigations into the potential of microbicides to select for reduced microbicide susceptibility in bacteria and induce cross-resistance to antibiotics have been largely

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conducted by evaluating susceptibility changes following exposure of bacteria to microbicides in simple aqueous solution (17). In such experiments, susceptibility of the exposed bacteria has been reported to decrease for certain combinations of bacterium and microbicide either transiently or stably (26). In the real world however microbicides are deployed in complex formulations containing sequestrants, surfactants and other compounds. Recent investigations indicate that the formulation of microbicides can significantly enhance antibacterial potency and that decreases in microbicide susceptibility after sub-lethal microbicide exposure may be significantly lower in frequency and extent when the microbicides are incorporated into formulations reflecting application in the real world (24, 33). This highlights the value of risk assessments that more accurately reflect the way microbicides are deployed. In the current investigation we have evaluated whether the formulation of microbicides additionally mitigates the development of antibiotic insusceptibility in bacteria.

In order to investigate whether the formulation of microbicides affects cross-resistance to antibiotics, we studied the induction of changes in antibiotic susceptibility in bacteria that had been repeatedly exposed, using a highly selective system arguably representing a worst case scenario, to microbicides in simple aqueous solution and in formulation with ingredients that are used in consumer products such as laundry detergents, hard surface disinfectants and personal care products (24). It should be noted that whilst the majority of microbicides tested are widely used in domestic cleaning products, the use of triclosan in Europe is generally restricted to applications where its utility is greatest, such as oral care.

Out of 288 microbicide-exposed bacteria, 28 organisms previously demonstrated a ≥4-fold decrease in microbicide susceptibility (18 organisms adapted to microbicides following exposure to simple aqueous solutions and 10 to microbicides in formulation). These were further evaluated for changes in antibiotic susceptibility in the current study. The difference in

316 the numbers of test bacteria between treatment groups results from the mitigating effects that
317 the formulation of microbicides had on the development of microbicide insusceptibility.
318 Increases in antibiotic susceptibility occurred at higher frequency following exposure to
319 simple solutions in comparison to formulations (20% *v* 12%) whilst 22% became significantly
320 less susceptible to the antibiotics regardless of formulation. Whilst both increases and
321 decreases in antibiotic susceptibility were observed in the test bacteria after exposure to
322 microbicide/formulation, no bacterium became resistant according to published BSAC
323 breakpoints.

324 Changes in antibiotic susceptibility varied between the test antibiotics, bacteria and the
325 microbicides that the bacteria had been previously adapted to, suggesting little correlative
326 effect between the different variables. One positive correlation was however observed
327 between the β-lactam antibiotics ampicillin and cephalothin (Table 2). In this case,
328 microbicide exposure could have altered alteration transpeptidase expression or otherwise
329 influenced cell wall permeability, subsequently impacting on the efficacy of these antibiotics
330 which target cell wall synthesis.

331 In some cases, bacterial antibiotic susceptibility was increased following microbicide
332 exposure. It is notable that such "cross-susceptibility" was associated with adaptation to at
333 least some microbicides for all antibiotics except ampicillin (Fig. 1). The phenomenon of
334 "cross-susceptibility" has been observed in several previous investigations (17, 22, 34, 35)
335 where links between antibiotics and decreased microbicide susceptibility in bacteria have
336 been demonstrated *in vitro* (14, 17). In a recent study, exposure of *Burkholderia cepacia* to
337 low concentrations of either CHX or BAC resulted in variable reductions in antibiotic
338 susceptibility (36). CHX exposure was reportedly associated with significant decreases in
339 susceptibility to ceftazidime, ciprofloxacin and imipenem, whilst short-term exposure to BAC

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resulted in significant decreases in ceftazidime, ciprofloxacin and meropenem susceptibility. These effects were however highly variable between biological replicates in a manner suggestive of stochastic effects. In another recent investigation, six *S. aureus* strains including methicillin-resistant *S. aureus* were repeatedly exposed to triclosan. Susceptibility to triclosan was significantly decreased in all exposed bacteria, whereas antibiotic susceptibility was significantly increased in the majority of cases. Whilst the reasons for cross-susceptibility have not been elucidated, they are likely to include general fitness costs of adaptation and transient cellular damage as previously hypothesized (37).

Mechanisms of cross-resistance have been more extensively investigated and include non-specific reductions in cell permeability, active efflux of the compound from the bacterial cell or acquired mutations in shared target sites (14, 17). Antibiotics such as aminoglycosides enter the cell through a mechanism of self-promoted uptake (38) whereby they displace cations in the bacterial cell envelope leading to the reorganisation of lipopolysaccharide, which may facilitate antibiotic entry. This mechanism of self-promoted uptake mirrors that of polymeric biguanides, such as PHMB and CHX (39) which has led to the question as to whether any adaptation to reduce biguanide uptake may have a resulting effect on the uptake of aminoglycosides into the bacterial cell. The current investigation included the evaluation of any changes in susceptibility to the aminoglycoside antibiotic kanamycin in bacteria that had previously shown reduced susceptibility to both CHX and PHMB. However, we found no evidence of a systematic effect of this sort (indeed adaptation to CHX typically led to an increase in susceptibility to kanamycin; Fig. 1) and only the PHMB adapted *E. coli* drain isolate showed any significant reduction in antibiotic susceptibility (Table 1).

Cross-resistance between quaternary ammonium compounds (QACs), such as BAC and DDAC and antibiotics has been attributed to the expression of broad-range efflux systems

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364 capable of removing both the microbicide as well as certain antibiotics from the bacterial cell
365 (40-42). It has additionally been noted that genes encoding QAC-specific efflux pumps such
366 as *qacA/B* may be detected on plasmids bearing β-lactamases in certain clinical isolates,
367 suggesting another cause for correlation between QACs and penicillins, such as ampicillin
368 (43). Furthermore, the *qacE* gene has been detected in the 3' conserved structure (3'-CS) of
369 certain class 1 integrons found in many Gram-negative bacteria. Class 1 integrons often
370 contain multiple antibiotic resistance genes and since they are commonly located on plasmids
371 this makes them highly mobile via the action of plasmid-mediated conjugation. This
372 consequentially facilitates the dissemination of both QAC and antibiotic resistance genes
373 through a population via horizontal gene transfer (44). Our data indicate that 20% of bacterial
374 isolates with reduced BAC and DDAC susceptibility in addition to 40% and 10% of isolates
375 with reduced DDAC or BAC formulation susceptibility, were also significantly reduced in
376 their antibiotic susceptibility. Linear mixed effect modelling revealed that the formulation of
377 BAC conferred a moderate protective effect on the development of antibiotic cross-resistance
378 (Fig. 2), possibly suggesting a regulatory impact of the formulation excipients on the
379 induction of the aforementioned efflux mechanisms, due to non-specific effects on cell
380 permeability or through other cellular changes.

381 Triclosan exposure may select for mutations in the target enzyme *fabI*, an enoyl-acyl carrier
382 protein reductase that participates in bacterial fatty acid synthesis (45). There has been
383 concern over the induction of cross-resistance between triclosan and therapeutic agents that
384 also share this target enzyme, such as isoniazid used to treat *Mycobacterium tuberculosis*.
385 Cross-resistance between triclosan and certain antibiotics has been reported in *P. aeruginosa*
386 and is largely believed to be due to increased expression of the MexAB-OprM efflux system
387 (14). In the current investigation, data show reductions in ciprofloxacin susceptibility in *S.*
388 *aureus* and the *E. coli* drain isolate together with reductions in ampicillin susceptibility in *S.*

389 *aureus* and *C. sakazakii* after repeated triclosan exposure, which may potentially be mediated
390 through regulation of efflux or cell permeability.

391 Whilst the induction of cross-resistance between microbicides and antibiotics has been
392 previously investigated, little information is available concerning any effect of incorporation
393 of microbicides into formulations containing surfactants and sequestrants on antibiotic
394 susceptibility in adapted bacteria. Data presented here indicate that both decreases and
395 increases in antibiotic susceptibility can occur in bacteria following exposure to microbicides
396 in simple solution and in formulations using a highly selective system. A rigorous statistical
397 analysis demonstrated that formulation significantly affected the development of cross-
398 resistance but that this was variable with the only consistently identified formulation effect
399 being a small increase in susceptibility across antibiotics in strains adapted to the formulated,
400 relative to the unformulated version of the microbicide benzalkonium chloride.

401 In conclusion, whilst both increases and decreases in antibiotic susceptibility were observed in
402 microbicide and formulation adapted bacteria, these were not sufficient to confer clinical
403 resistance according to published BSAC breakpoints.

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409 ## TRANSPARENCY DECLARATION

410 Alejandro Amézquita is an employee of Unilever. Peter McClure was an employee of
411 Unilever when this project was initiated. All other authors: none to declare.

REFERENCES

1. **Pereira M, Vieira M, Beleza V, Melo L.** 2001. Comparison of two biocides-carbamate and glutaraldehyde-in the control of fouling in pulp and paper industry. Environ Technol **22:**781-790.

2. **Barbolt TA.** 2002. Chemistry and safety of triclosan, and its use as an antimicrobial coating on Coated VICRYL* Plus Antibacterial Suture (coated polyglactin 910 suture with triclosan). Surg Infect (Larchmt) **3 Suppl 1:**S45-53.

3. **Bibbo C, Patel D, Gehrmann R, Sheldon L.** 2005. Chlorhexidine provides superior skin decontamination in foot and ankle surgery: a prospective randomized study. Clin Orthop Relat Res **438**:204-208.

4. **Abreu AC, Tavares RR, Borges A, Mergulhão F, Simões M.** 2013. Current and emergent strategies for disinfection of hospital environments. J Antimicrob Chemother **68:**2718-2732.

5. **McBain AJ, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Gilbert P.** 2003. Effects of a chlorhexidine gluconate-containing mouthwash on the vitality and antimicrobial susceptibility of *in vitro* oral bacterial ecosystems. Appl Environ Microbiol **69:**4770-4776.

6. **Koburger T, Hubner NO, Braun M, Siebert J, Kramer A.** Standardized comparison of antiseptic efficacy of triclosan, PVP-iodine, octenidine dihydrochloride, polyhexanide and chlorhexidine digluconate. J Antimicrob Chemother **65**:1712-1719.

7. **Best M, Kennedy M, Coates F.** 1990. Efficacy of a variety of disinfectants against *Listeria* spp. Appl Environ Microbiol **56:**377-380.

8. **McBain A, Gilbert P.** 2001. Biocide tolerance and the harbingers of doom. Int Biodeterior biodegradation **47:**55-61.

9. **Maillard J-Y.** 2010. Emergence of bacterial resistance to microbicides and antibiotics. Microbiol Aust **31:**159-164.

10. **Maillard J-Y.** 2007. Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern? J Hosp Infect **65:**60-72.

11. **Karatzas KA, Webber MA, Jorgensen F, Woodward MJ, Piddock LJ, Humphrey TJ.** 2007. Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. J Antimicrob Chemother **60:**947-955.

12. **Tattawasart U, Maillard JY, Furr JR, Russell AD.** 1999. Development of resistance to chlorhexidine diacetate and cetylpyridinium chloride in *Pseudomonas stutzeri* and changes in antibiotic susceptibility. J Hosp Infect **42:**219-229.

13. **Webber MA, Whitehead RN, Mount M, Loman NJ, Pallen MJ, Piddock LJ.** 2015. Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. J Antimicrob Chemother **70**:2241-2248.

14. **Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP.** 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: Exposure of a susceptible mutant strain to triclosan

selects nfxB mutants overexpressing MexCD-OprJ. Antimicrob Agents Chemother **45:**428-432.

15. **Winder CL, Al-Adham ISI, Abdel Malek SMA, Buultjens TEJ.** 2000. Outer membrane protein shifts in biocide resistant *Pseudomonas aeruginos*a PAO1. J Appl Microbiol **89:**289-295.

16. **Bloomfield SF, Arthur M.** 1994. Mechanisms of inactivation and resistance of spores to chemical biocides. J Appl Microbiol **76:**91S-104S.

17. **Walsh SE, Maillard J-Y, Russell A, Catrenich C, Charbonneau D, Bartolo R.** 2003. Development of bacterial resistance to several biocides and effects on antibiotic susceptibility. J Hosp Infect **55:**98-107.

18. **Oggioni MR, Furi L, Coelho JR, Maillard J-Y, Martínez JL.** 2013. Recent advances in the potential interconnection between antimicrobial resistance to biocides and antibiotics. Exp Rev Anti Infect Ther **11:**363-366

19. **Cottell A, Denyer S, Hanlon G, Ochs D, Maillard J-Y.** 2009. Triclosan-tolerant bacteria: changes in susceptibility to antibiotics. J Hosp Infect **72:**71-76.

20. **Maillard J-Y.** 2005. Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. Ther Clin Risk Manag **1:**307-320.

21. **Morrissey I, Oggioni MR, Knight D, Curiao T, Coque T, Kalkanci A, Martinez JL, Consortium B.** 2014. Evaluation of epidemiological cut-off values indicates that biocide resistant subpopulations are uncommon in natural isolates of clinically-relevant microorganisms. PLoS One **9**.1

22. **Forbes S, McBain AJ, Felton-Smith S, Jowitt TA, Birchenough HL, Dobson CB.** 2013. Comparative surface antimicrobial properties of synthetic biocides and novel human apolipoprotein E derived antimicrobial peptides. Biomaterials **34:**5453-5464.

23. **Ledder RG, Gilbert P, Willis C, McBain AJ.** 2006. Effects of chronic triclosan exposure upon the antimicrobial susceptibility of 40 *ex-situ* environmental and human isolates. J Appl Microbiol **100:**1132-1140.

24. **Cowley N, Forbes S, Amézquita A, McClure P, Humphreys G, McBain AJ.** 2015. The effect of formulation on microbicide potency and mitigation of the development of bacterial insusceptibility. Appl Environ Microbiol **20**:7330-7338.

25. **Marshall BM, Robleto E, Dumont T, Levy SB.** 2012. The frequency of antibiotic-resistant bacteria in homes differing in their use of surface antibacterial agents. Curr Microbiol **65:**407-415.

26. **Forbes S, Dobson CB, Humphreys GJ, McBain AJ.** 2014. Transient and sustained bacterial adaptation following repeated sublethal exposure to microbicides and a novel human antimicrobial peptide. Antimicrob Agent Chemother **58:**5809-5817.

27. **Moore LE, Ledder RG, Gilbert P, McBain AJ.** 2008. *In vitro* study of the effect of cationic biocides on bacterial population dynamics and susceptibility. Appl Environ Microbiol **74:**4825-4834.

28. **Andrews JM.** 2001. BSAC standardized disc susceptibility testing method. J Antimicrob Chemother **48:**43-57.

29. **Pagel M.** 1999. Inferring the historical patterns of biological evolution. Nature **401:**877-884.

30. **Pinheiro J, Bates D.** 2006. Mixed-effects models in S and S-PLUS. Springer Science & Business Media.

31. **Team RC.** 2015. R: A language and environment for statistical computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2013. Document freely available on the internet at: http://www.r-project. org.

32. **Paradis E.** 2011. Analysis of Phylogenetics and Evolution with R. Springer Science & Business Media.

33. **Knapp L, Amézquita A, McClure P, Stewart S, Maillard J-Y.** 2015. Development of a protocol for predicting bacterial resistance to microbicides. Appl Environ Microbiol **81:**2652-2659.

34. **Belavkin RV, Aston JA, Channon A, Aston E, Rash BM, Kadirvel M, Forbes S, Knight CG.** 2014. Mutation rate plasticity in rifampicin resistance depends on *Escherichia coli* cell–cell interactions. Nat Commun **5**.

35. **Forbes S, Latimer J, Bazaid A, McBain AJ.** 2015. Altered competitive fitness, antimicrobial susceptibility, and cellular morphology in a triclosan-induced small-colony variant of *Staphylococcus aureus*. Antimicrob Agent Chemother **59:**4809-4816.

36. **Knapp L, Rushton L, Stapleton H, Sass A, Stewart S, Amezquita A, McClure P, Mahenthiralingam E, Maillard JY.** 2013. The effect of cationic microbicide exposure against Burkholderia cepacia complex (Bcc); the use of *Burkholderia lata* strain 383 as a model bacterium. J Appl Microbiol **115:**1117-1126.

37. **McBain AJ, Ledder RG, Sreenivasan P, Gilbert P.** 2004. Selection for high-level resistance by chronic triclosan exposure is not universal. J Antimicrob Chemother **53:**772-777.

38. **Hancock RE.** 1981. Aminoglycoside uptake and mode of action—with special reference to streptomycin and gentamicin I. Antagonists and mutants. J Antimicrob Chemother **8:**249-276.

39. **Gilbert P, Pemberton D, Wilkinson DE.** 1990. Synergism within polyhexamethylene biguanide biocide formulations. J Appl Microbiol **69:**593-598.

40. **Chen J, Kuroda T, Huda MN, Mizushima T, Tsuchiya T.** 2003. An RND-type multidrug efflux pump SdeXY from *Serratia marcescens.* J Antimicrob Chemother **52:**176-179.

41. **Levy SB.** 2002. Active efflux, a common mechanism for biocide and antibiotic resistance. Journal of applied microbiology **92:**65S-71S.

42. **Maseda H, Hashida Y, Konaka R, Shirai A.** 2009. Mutational up-regulation of an RND-type multidrug efflux pump, SdeAB, upon exposure to a biocide, cetylpyridinium chloride, and antibiotic resistance in *Serratia marcescens*. Antimicrob Agent Chemother **53:**5230-5235.

43. **Lyon BR, Skurray R.** 1987. Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. Microbiol Rev **51:**88.

44. **Paulsen I, Littlejohn T, Rådström P, Sundström L, Sköld O, Swedberg G, Skurray R.** 1993. The 3'conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob Agent Chemother **37:**761-768.

45. **McMurry LM, Oethinger M, Levy SB.** 1998. Triclosan targets lipid synthesis. Nature **394:**531-532.

Table 1. Antibiotic susceptibility of bacterial isolates that showed a ≥ 4-fold decrease in microbicide/formulation susceptibility following exposure to microbicides in simple aqueous solution or formulated with surfactants and sequestrants.

		Ciprofloxacin			Kanamycin			Cephalothin			Ampicillin			Tetracycline		
		UE	UF	F	UE	UF	F	UE	UF	F	UE	UF	F	UE	UF	F
Microbicide	Bacterium	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14
BAC	*S. aureus*†	22	**14 (0.5)**	**18 (0.5)**	17 (1.5)	**14 (0.6)**	17 (0.5)	45 (0.5)	**43**	45	47 (0.5)	45 (0.5)	46	26 (0.5)	25 (0.5)	27 (0.5)
	E. coli†	29 (1.5)	**31**	31 (0.5)	15 (1.2)	**12 (0.5)**	14 (0.4)	18 (0.5)	**16 (2.1)**	18	21	22 (0.5)	21	21 (0.5)	21 (0.5)	20 (0.5)
	P. aeruginosa†	25 (1.5)	25	na	ns	ns	na	ns	ns	na	ns	ns	na	ns	ns	na
DDAC	*P. aeruginosa*†	25 (1.5)	25	**28 (0.6)**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	*A. baumanii**	19	**27**	na	19	**21**	na	ns	ns	na	ns	ns	na	15	**13**	na
	*E. coli**	37	**42 (1.5)**	**40 (0.6)**	14	**18**	**11**	19	24 (2.1)	**15 (0.5)**	25	26 (1.5)	**21 (0.6)**	20	**11 (0.5)**	**11 (0.5)**
CHX	*S. aureus*†	22	**19 (0.5)**	na	17 (1.5)	18	na	45 (0.6)	45 (0.5)	na	47 (0.5)	**29 (1)**	na	26 (0.6)	**35 (2.2)**	na
	E. coli†	29 (1.5)	**35 (0.5)**	na	15 (1.2)	16 (0.5)	na	18 (0.5)	20 (2.1)	na	21	**24 (0.5)**	na	21 (0.5)	23 (1.5)	na
PHMB	*S. aureus*†	22	**20 (0.5)**	**21**	17 (1.5)	17 (1.2)	16 (0.5)	45 (0.6)	45 (0.5)	45	47 (0.5)	**35 (0.5)**	45 (1.5)	26 (0.6)	**36 (1.5)**	25 (0.5)
	E. coli†	29 (1.5)	29	na	15 (1.2)	16 (0.5)	na	18 (0.5)	18 (2.1)	na	21	20 (1.5)	na	21 (0.5)	22 (0.5)	na
	P. aeruginosa†	25 (1.5)	25	25 (0.9)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	E. faecalis†	ns	ns	ns	ns	ns	ns	12	13 (0.5)	12 (0.5)	33	33	33 (1.3)	8	8	9 (0.5)
	*E. coli**	37	**35**	na	14	**12 (1.5)**	na	19	16	na	25	**20 (0.5)**	na	20	**24**	na
DMDM	*E. coli* *	37	**28 (0.6)**	na	14	**12 (1.5)**	na	19	18 (2.2)	na	25	25 (0.5)	na	20	20 (0.5)	na
Thymol	*E. coli*†	29 (1.5)	na	**33**	15 (1.2)	na	**14**	18 (0.5)	na	**19**	21	na	21	21 (0.5)	na	**20**
	*P. putida**	27	na	**19.5 (0.5)**	30	na	**27 (0.5)**	ns	na	ns	ns	na	ns	14	na	12 (2.1)
	*A. baumanii**	19	na	**33 (0.5)**	19	na	**22**	ns	na	ns	ns	na	ns	15	na	**16 (0.5)**
Triclosan	*S. aureus*†	22	**21 (0.5)**	na	17 (1.5)	**21 (0.5)**	na	45 (0.5)	**51 (2.5)**	na	47 (0.5)	**44 (0.5)**	na	26 (0.5)	**34**	na
	E. coli†	29 (1.5)	**41 (1.5)**	na	15 (1.2)	13 (0.5)	na	18 (0.5)	18 (0.5)	na	21	**28 (0.5)**	na	21 (0.6)	20 (1.5)	na
	*C. sakazakii**	28	**32 (0.6)**	na	17	**20 (0.5)**	na	11	**12**	na	25	**21 (0.5)**	na	17	17 (0.5)	na
	E. coli *	37	**35**	na	14	15 (1.3)	na	19	**20**	na	25	24 (1.2)	na	20	23 (2.1)	na

Data show growth inhibition zones (mm) representative of antibiotic susceptibility before (P0) and after 14 passages (P14) in the presence of microbicide/formulation. Antibiotic zones of inhibition were determined before antimicrobial exposure (unexposed; UE) and after antimicrobial exposure to both unformulated (UF) (i.e. simple aqueoussolution) and formulated (F) (i.e. with surfactants and sequestrants) microbicides. †, non-drain isolates; *, drain isolates. Statistically significant changes are bold text ($P < 0.05$). Bacteria that did not undergo a ≥4-fold change in MBC were not assessed for changes in antibiotic susceptibility. Where data varied between biological replicates, standard deviations have been given in parentheses (n=6). Combinations of bacterium and antibiotic for which BSAC breakpoints are available are indicated in blue text. According to these, no susceptible bacterium became antibiotic resistant following microbicide adaptation.

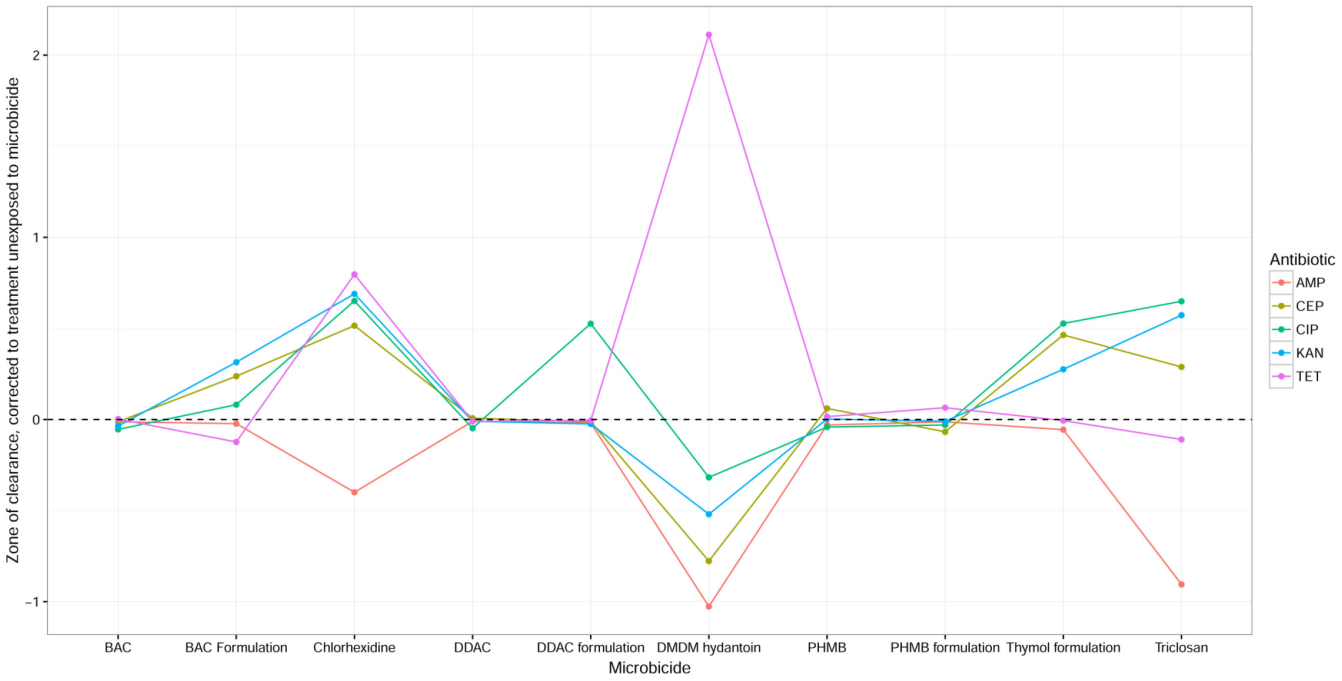

Fig. 1. Antibiotic susceptibility of strains adapted to different microbicides. The values plotted are the difference in the average zone of clearance across strains before and after adaptation to the given microbicide as estimated by the linear mixed effects model (arbitrary scale, see methods). i.e. values above zero indicate antibiotic cross-susceptibility arising from adaptation to microbicide and values below zero indicate cross-resistance. Points are connected for ease of comparison only. See footnote to Tables 1 and 2.

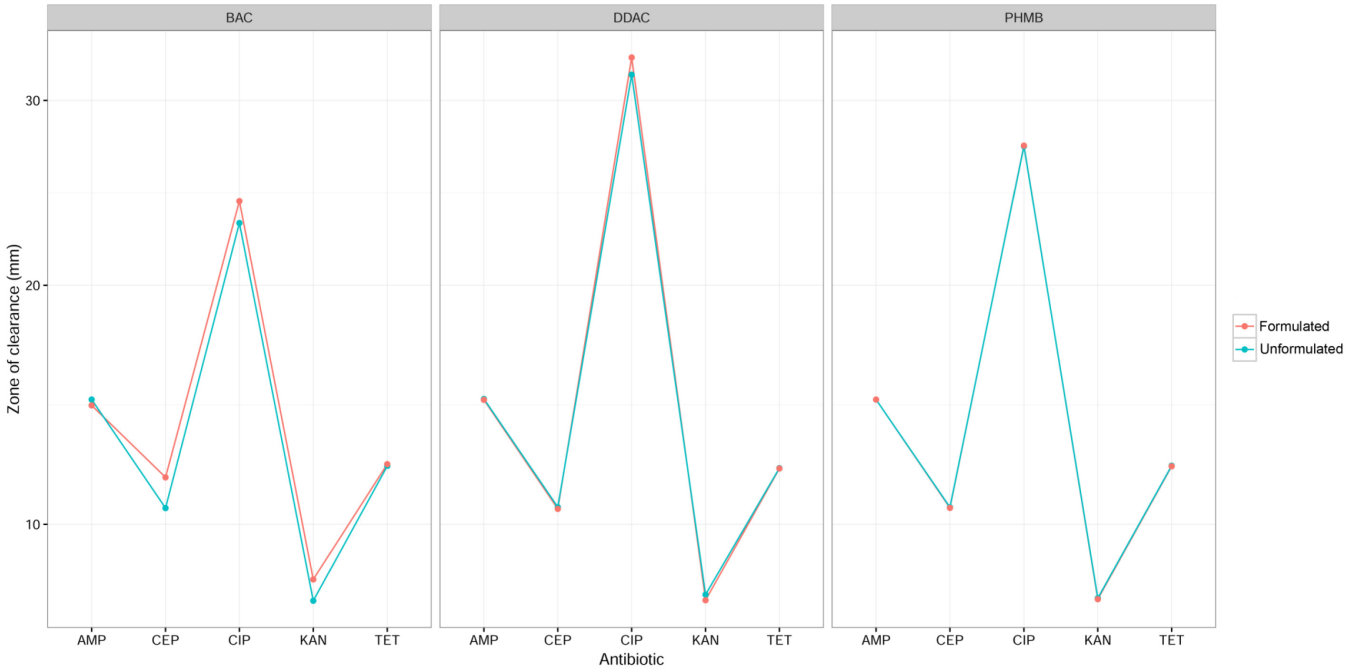

Fig. 2. Antibiotic susceptibility of strains exposed to different microbicides in formulation with surfactants and sequestrants) and simple aqueous solution (unformulated). A significant difference is only apparent for BAC. The values plotted are the average zone of clearance, in mm, as estimated in the linear mixed effects model (note the transformed scale as used by the model, see methods). See footnote to Table 1.

Table 2. Correlation across strains in the responses to different antibiotics in the linear mixed effects model.

	AMP	CEP	CIP	KAN	TET
AMP	1	0.95	-0.28	-0.08	0.54
CEP	0.95	1	-0.09	0.03	0.61
CIP	-0.28	-0.09	1	0.54	0.17
KAN	-0.08	0.03	0.54	1	0.73
TET	0.55	0.61	0.17	0.73	1
Key:					

A value of 1 indicates that all organisms respond in a perfectly correlated way to the two antibiotics indicated (either more or less sensitive to both), a value of -1 would indicate a perfect negative correlation with organisms that are more sensitive to one antibiotic. Amp, ampicillin; cep, cephalothin; cip, ciprofloxacin; kan, kanamycin; tet, tetracycline.