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The Effect of Formulation on Microbicide Potency and Mitigation of the Development of Bacterial Insusceptibility

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Running title: Bacterial susceptibility to microbicide formulations.

Key words: Microbicide, susceptibility, formulation, active

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Risk assessments into the potential for microbicides to select for reduced bacterial susceptibility have been based largely on data generated through the exposure of bacteria to microbicides in aqueous solution. Since microbicides are normally formulated with multiple excipients, we have investigated the effect of formulation on antimicrobial activity and the induction of bacterial insusceptibility. The susceptibilities of 9 species of bacteria (7 genera) were determined before and after repeated exposure (14 passages) using a previously validated gradient plating system, to the microbicides benzalkonium chloride, benzisothiozolinone, chlorhexidine, didecyldimethyl ammonium chloride, DMDM-hydantoin, polyhexamethylene biguanide, thymol and triclosan in aqueous solution (non-formulated) and in formulation with excipients often deployed in consumer products. Susceptibilities were also assessed following an additional 14 passages without microbicide to determine the stability of any susceptibility changes. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were on average 11-fold lower for formulated *vs.* non-formulated microbicides. After antimicrobial exposure, of 72 combinations of microbicide and bacterium, there were 19 ≥4-fold (mean 8-fold) increases in MIC for non-formulated and 8 ≥4-fold (mean 2-fold) increases in MIC for formulated microbicides. Furthermore, there were 20 ≥4-fold increases in MBC (mean 8-fold) for non-formulated and 10 ≥4-fold (mean 2-fold) increases in MBC for formulated microbicides. Susceptibility decreases fully or partially reverted back to pre-exposure values for 49% of MICs and 72% of MBCs after further passage. In summary, formulated microbicides exhibited greater antibacterial potency than unformulated actives and susceptibility decreases following repeated exposure were lower in frequency and extent.

INTRODUCTION

Microbicides are broad-spectrum chemical agents that inactivate microorganisms (1-3). They are widely deployed throughout healthcare (4-6), domestic (7, 8) and industrial environments (9-11) where their application includes antisepsis (12), hard surface disinfection (13) and pharmaceutical product preservation (14). They may also be incorporated into medical device coatings, for instance in sutures (15), wound dressings (16) and urinary catheters (17) to inhibit bacterial adhesion and subsequent biofilm formation.

It has been hypothesized that the use of microbicides could select for bacterial adaptation, resulting in reduced efficacy of the primary agent as well as potentially decreasing bacterial susceptibility to chemically-unrelated agents such as other microbicides and antibiotics (18). Whilst there have been reports documenting the laboratory selection of bacteria with decreased

62 microbicide sensitivity following repeated exposure to microbicides in highly selective
63 conditions, it remains unclear whether this commonly occurs in the environment (19-24).

64 The majority of studies reporting reductions in microbicide susceptibility have used the active
65 compound in aqueous solution with or without the addition of co-solvents such as DMSO (25)
66 or ethanol (26, 27). In real use however, microbicides are deployed in formulated products with
67 multiple excipients that may enhance potency. The potential effect of the formulation of
68 microbicides on reducing the development of bacterial insusceptibility has received little
69 research attention. Furthermore, despite the research effort that has been directed towards the
70 possible risk of induced microbicide insusceptibility, the stability of such susceptibility changes
71 has been investigated infrequently (24).
72

73 With the ultimate aim of developing realism-based approaches to risk assessment, the current
74 investigation evaluates the frequency, magnitude and reversibility of susceptibility changes that
75 may be induced by the repeated exposure of a range of bacteria to microbicides in aqueous
76 solution or in formulation. The microbicides selected reflect those frequently used in consumer
77 products such as laundry detergents, hard surface disinfectants and personal care products.
78 Planktonic susceptibilities (MIC, MBC) and minimum biofilm eradication concentrations
79 (MBEC) were determined before and after repeated exposure to sub-lethal concentrations of the
80 microbicides benzalkonium chloride (BAC), benzisothiozolinone (BIT), chlorhexidine (CHX),
81 didecyldimethyl ammonium chloride (DDAC), glydant (DMDM hydantoin),
82 polyhexamethylene biguanide (PHMB), thymol, and triclosan in aqueous solution and in
83 formulation with commonly used sequestrants and surfactants. Bacteria were also passaged
84 further in the absence of any antimicrobial to determine the stability of any observed change in
85 susceptibility.

METHODS

Bacteria. *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, and *Escherichia coli* ATCC 25922 were obtained from Oxoid (Basingstoke, UK). *Acinetobacter baumanii* (Accession number: JX966428.1), *Pseudomonas putida* (Accession number: JQ968690.1), *Moraxella osloensis* (Accession number: AB643597.1), *Escherichia coli* (Accession number: CP003034.1) and *Cronobacter sakazakii* (Accession number: HQ880381.1) were isolated from a domestic kitchen drain biofilm. *Enterococcus faecalis* (Accession number KJ818115.1) was provided by Angela Oates, The University of Manchester.

Chemical Reagents and Growth Media. Bacteriological growth media was purchased from Oxoid (Basingstoke, UK). All other chemical reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Bacterial growth media was sterilized at 121°C and 15 psi for 15 min prior to use. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis* were cultured on Tryptone Soya Agar and Broth. *Acinetobacter baumanii, Pseudomonas putida*, *Moraxella osloensis* and *Cronobacter sakazakii* were grown on Wilkins Chalgren agar and broth containing 2% sucrose. All bacteria were incubated aerobically at 37˚C for 18h unless stated otherwise.

Antimicrobial actives: benzalkonium chloride, chlorhexidine, thymol and triclosan were purchased from Sigma-Aldrich (Dorset, UK). Didecyldimethyl ammonium chloride (50% v/v) was purchased from Merck Millipore (Durham, UK). Vantocil (a 20% v/v aqueous solution of PHMB) was obtained from Arch Chemicals Inc. (Manchester, UK). Glydant (DMDM hydantoin) was obtained from Lonza (Bishop's Stortford, UK). All microbicides were tested in aqueous solution as previously described (27) and in formulation, at concentrations reflective of their normal deployment in consumer products. BAC, CHX, DDAC, DMDM hydantoin, PHMB and thymol were prepared at 1% (v/v) in a general purpose cleaner. Triclosan was formulated

into a laundry detergent at 0.0066% (w/v). Benzisothiozolinone was formulated into a laundry detergent at 0.02% (v/v).

Exposure of Bacteria to Sub-lethal Concentrations of Microbicides as active and formulation. A previously validated system (20, 25) was used to generate reproducible *c.* 100-fold antimicrobial concentration gradients on Tryptone Soya Agar plates using a spiral plater (Whitley Automated Spiral Plater, Don Whitley Scientific, Shipley, UK). Initial MIC antimicrobial stock solutions (50µl) were deposited on the agar surface. Plates were dried for 1h at room temperature prior to radial deposition of bacterial pure cultures and then incubated (4d; 37˚C) in a static aerobic incubator. After incubation, growth observed at the highest microbicide concentration was aseptically removed and streaked onto a fresh plate containing the same antimicrobial concentration gradient. Where growth was observed across the whole antimicrobial gradient, a new plate produced with a five times higher microbicide concentration was used[25]. This process was repeated until 14 passages had occurred (P14). Bacteria that exhibited ≥4-fold changes in MIC, MBC or MBEC were then passaged a further 14 times in the absence of any antimicrobial (X14) to ascertain the stability of adaptation. Bacteria at P0, P14 and X14 were archived for subsequent MIC and MBC testing. Susceptibility testing (MIC, MBC, MBEC) was performed in two separate experiments each with three technical replicates.

Determination of bacterial Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC). MIC values were determined using the microdilution method as described previously (28). Briefly, overnight bacterial cultures were adjusted to an OD_{600} of 0.8 and diluted 1 in 100 in Tryptone Soya Both or Wilkins Chalgren Broth with 2% sucrose in a 96-well microtiter plate containing doubling dilutions of the relevant microbicide. Plates were incubated at 37ºC (24h) with agitation (100rpm). The MIC was defined as the lowest concentration for which bacterial growth did not occur. Growth was viewed as turbidity (600nm) in comparison to an uninoculated well (negative control) and was detected

using a microtiter plate reader (Anthos HTII; Anthos-Labtec Instruments. Salzburg. Austria). MBCs were determined as stated previously (25), in brief aliquots (10µl) from wells exhibiting no turbidity were transferred to sterile Tryptone Soya Agar or Wilkins Chalgren Agar prior to 4d incubation at 37ºC to determine the minimum bactericidal concentration (MBC) (25). The MBC was defined as the lowest concentration of microbicide at which no growth occurred after 4d of incubation.

Determination of Minimum Biofilm Eradication Concentrations. Single species biofilms were grown on the pegs of a Calgary Biofilm Device (CBD) (29). To produce inocula for biofilm susceptibility testing, single colonies of test bacteria were inoculated into 10ml of sterile Tryptone Soya Broth or Wilkins Chalgren Broth with 2% sucrose and incubated at 37˚C in a shaking aerobic incubator (100rpm) for 18h. Cultures were diluted to an OD_{600} of 0.8, then further diluted 1:100 using fresh growth medium. 100µl of bacterial inoculum was added to each well of the CBD base, plates were then incubated at 37˚C and 30 rpm for 48h to allow biofilm formation on the pegs. Doubling dilutions for microbicides (150µl) were prepared in sterile broth across a 96 well microtiter plate. Biofilms were exposed to antimicrobials and incubated for 24h at 37˚C and 100rpm. After incubation the lid was transferred to a 96-well plate containing 200µl of sterile broth and was incubated for 24h at 37˚C and 100rpm. Minimum biofilm eradication concentrations (MBECs) were determined as the lowest concentration for which bacterial growth did not occur after 18h of incubation. Growth was viewed as turbidity in comparison to an uninoculated well (negative control) and was detected using a microtiter plate reader (BioTek, Bedfordshire, UK).

RESULTS

Two main variables describe data associated with the selection of decreased susceptibility by exposure to microbicides in the current study; i) the frequency of susceptibility decreases greater

than two-fold (25) for multiple test bacteria and microbicides and ii) the extent of susceptibility changes for each combination of bacterium and microbicide.

Repeated exposure to the microbicide-containing formulations resulted in a lower frequency of susceptibility reductions than did exposure to the same microbicide in aqueous solutions and, where decreases in susceptibility did occur; these were generally smaller for formulated microbicides. All individual values for bacterial susceptibility before, during and after microbicide exposure have been given in Tables 1-8. However, due to the large number of combinations of bacterium and antimicrobial that were tested, the extent of susceptibility has also been expressed as mean values in the following section.

After repeated exposure to unformulated microbicides there were 19 ≥4-fold increases in MIC (1 of which fully reverted back to pre-exposure values after subsequent passage in the absence of microbicide, 13 of which partially reverted and 5 which did not revert; average increase in MIC (P0 to P14) was 11-fold across the test panel of bacteria and microbicides). There were 20 increases in MBC (2 fully, 11 partially and 7 non-revertible; average 8-fold increase) and 17 increases in MBEC (7 fully, 6 partially and 4 non- revertible; average 4-fold increase) after microbicide exposure (Tables 1-8). After exposure to microbicide containing formulations there were 8 ≥4-fold increases in MIC (2 fully and 6 non-revertible; average 2-fold increase), 10 increases in MBC (3 fully, 5 partially and 2 non-revertible; average 2-fold increase) and 16 increases in MBEC (5 fully, 8 partially and 3 non-revertible; average 3-fold increase) (Tables 1-8). In terms of antimicrobial potency, when comparing the formulated to non-formulated microbicides across the test panel of bacteria we saw an approximately 11-fold lower MIC/ MBC and 3-fold lower MBEC for the unexposed (P0) bacterial isolates. For the P14 isolates we

observed an approximately 35-fold lower MIC, 36-fold lower MBC and 4-fold lower MBEC (Tables 1-8).

Benzalkonium Chloride. All test bacteria, with the exception of *M. osloensis*, *C. sakazakii* and the *E. coli* drain isolate exhibited a ≥4 fold increase in MIC after exposure to BAC (Table 1). Increases in MBC, whilst generally smaller than those in MIC, were also observed at ≥4 fold *for S. aureus, E. coli* and *P. aeruginosa*. Furthermore ≥4 fold increases in MBEC occurred for *S. aureus* and *E. faecalis* after BAC exposure. After growth in the absence of BAC, subsequent full or partial reversion in MIC, MBC or MBEC occurred for all test bacteria with the exception of *E. coli* and *P. aeruginosa* (MIC and MBC). In contrast, after exposure to the BAC formulation only *S. aureus*, *E. coli*, *P. aeruginosa* and *A. baumanii* showed a ≥4 fold increase in MIC with *S. aureus* and *E. coli* also demonstrating a ≥4 fold increase in MBC. *S. aureus*, *E. faecalis* and *P. aeruginosa* also exhibited a ≥4 fold increase in MBEC after exposure to BAC formulation. After recovery in the absence of BAC formulation only *S. aureus* demonstrated any reversion in susceptibility (MBEC).

Benzisothiozolinone (BIT). No bacterium displayed a substantial change in susceptibility (≥4 fold MIC, MBC or MBEC) to BIT or to BIT formulation after long-term exposure to the respective agent (Table 2).

Chlorhexidine. After repeated exposure to chlorhexidine both *S. aureus* and *E. coli* showed ≥4 fold increases in MIC and MBC which partially reverted in the absence of the microbicide (Table 3). *P. aeruginosa* demonstrated a ≥4 fold increase in MIC which did not revert after regrowth in a chlorhexidine free environment. *E. faecalis* and *M. osloensis* exhibited ≥4 fold increases in MBEC, which partially and fully reverted in the absence of chlorhexidine respectively. In contrast, after exposure to chlorhexidine formulation no bacterium exhibited a ≥4 fold decrease in susceptibility at MIC, MBC or MBEC level.

Didecyldimethyl Ammonium Chloride. After repeated DDAC exposure *P. aeruginosa, A. baumanii* and the *E .coli* drain isolate exhibited a ≥4 fold increase in MBC, of which *P. aeruginosa* fully reverted whilst *A. baumanii* and *E. coli* partially reverted following repeated growth the absence of DDAC. *S. aureus. E. coli, E. faecalis* and the *E. coli* drain isolate all exhibited a ≥4 fold increase in MBEC, out of which *E. faecalis* and the *E. coli* drain isolate partially reverted, *E. coli* fully reverted and *S. aureus* did not revert back to pre-exposure values following growth in the absence of the microbicide (Table 4). After exposure to the DDAC-containing formulation, *P. aeruginosa* and the *E. coli* drain isolate exhibited a ≥4 fold increase in MBC, out of which *E. coli* partially reverted and *P. aeruginosa* fully reverted after passage in an antimicrobial free environment. In agreement with the changes in MBEC observed after exposure to DDAC active, *S. aureus, E. coli, E. faecalis* and the *E. coli* drain isolate also showed a ≥4 fold increase in MBEC after exposure to DDAC formulation. MBEC values partially reverted for both *E. coli* isolates and for *E. faecalis* but did not revert for *S. aureus* after recovery in the absence of DDAC.

Glydant (DMDM Hydantoin). The *E. coli* drain isolate exhibited a ≥4 fold increase in MBC after repeated exposure to DMDM hydantoin; this susceptibility decrease fully reverted in the absence of the microbicide (Table 5). Comparatively after exposure to DMDM hydantoin formulation both *E. coli* isolates as well as *C. sakazakii* showed a ≥4 fold increase in MBEC, all of which fully reverted in an antimicrobial free environment.

Polyhexamethylene Biguanide. *S. aureus, E. faecalis M. osloensis* and *A. baumanii* exhibited a ≥4 fold increase in MIC after PHMB exposure out of which *M. osloensis* and *A. baumanii* fully reverted and *S. aureus* and *E. faecalis* partially reverted after growth in the absence of PHMB (Table 6). *S. aureus, E. coli, P. aeruginosa, E. faecalis,* and the *E. coli* drain isolate demonstrated a ≥4 fold increase in MBC out of which *S. aureus*, *E. faecalis* and the *E. coli* drain isolate showed partial reversion and *E. coli* and *P. aeruginosa* showed no reversion to

pre-exposure values in the absence of PHMB. After PHMB exposure, *S. aureus, E. faecalis, A. baumanii, C. sakazakii,* and the *E. coli* drain isolate also displayed a ≥4 fold increase in MBEC, which fully reverted for *S. aureus, A. baumanii* and *E. coli* drain isolate, and partially reverted for *E. faecalis* and *C. sakazakii* after re-growth in the absence of PHMB. After exposure to PHMB formulation *S. aureus, E. faecalis* and *P. aeruginosa* showed substantial changes in their PHMB susceptibility displaying ≥4 fold increases in MBC all of which fully or partially reverted in the absence of the antimicrobial formulation. *S. aureus* and *E. faecalis* also exhibited a ≥4 fold increase in MBEC after exposure to PHMB formulation, all of which partially reverted back to pre-exposure values after regrowth in the absence of the formulation.

Thymol. After long-term thymol exposure none of the bacterial isolates showed a ≥4 fold decrease in thymol susceptibility at MIC, MBC or MBEC level (Table 7). After exposure to the thymol-containing formulation, *E. coli* and *A. baumanii* both underwent ≥4 fold increases in MBC whilst *P. putida* demonstrated a ≥4 fold increase in MIC and MBC, all of which partially reverted in the absence of thymol formulation. Furthermore, both *E. coli* isolates showed a ≥4 fold increase in MBEC, which partially reverted after growth in the absence of thymol formulation.

Triclosan. All bacterial isolates, with the exception of *E. faecalis, A. baumanii* and *P. aeruginosa*, which is non-susceptible to triclosan, demonstrated an increase in MIC after repeated triclosan exposure, none of which fully reverted back to pre-exposure levels after regrowth in the absence of triclosan (Table 8). All isolates apart from *P. aeruginosa, A. baumanii* and *P. putida* showed a ≥4 fold increase in MBC out of which *C. sakazakii* and the *E. coli* drain isolate showed partial reversion, whilst the others showed no reversion after passage in the absence of triclosan. Both *E. coli* isolates in addition to *C. sakazakii, E. faecalis* and *A. baumanii* showed ≥4 fold increase in MBEC after repeated triclosan exposure out of which *C. sakazakii* and *E. faecalis* did not revert and both *E. coli* isolates completely reverted in the

absence of the microbicide. In comparison after exposure to triclosan formulation only the *E. coli* isolates and *P. aeruginosa* showed ≥4 fold increase in MIC, which fully reverted for *P. aeruginosa* but did not revert for either *E. coli* strain in the absence of triclosan formulation. MBECs increased ≥4 fold for *S. aureus* and *E. faecalis* but fully reverted for both bacteria after regrowth in the absence of triclosan formulation.

DISCUSSION

The majority of investigations into the potential of microbicides to select for changes in bacterial susceptibility have been conducted by exposing pure cultures of bacteria to microbicides as pure actives in aqueous solution or in simple formulations (aqueous solutions containing the active and in some studies, cosolvents such as DMSO (25) or ethanol (27)). It has been hypothesized that formulated products may interact with bacteria in a manner that is distinct from aqueous solutions (28, 30) potentially reducing the frequency and extent of susceptibility reductions. Whilst numerous studies have evaluated the antimicrobial potency of formulated microbicides (3, 31, 32), to our knowledge there are no studies in the literature that have compared the effects of repeated bacterial exposure to microbicides in aqueous solution and in complex formulation, for a range of bacteria and microbicides. In the current investigation therefore, we have evaluated the effect of the formulation of microbicides on antimicrobial potency and on the mitigation of bacterial insusceptibility for a selection of bacterial isolates and microbicides encompassing biguanides, quaternary ammonium compounds, phenolics, isothiazolinones, formaldehyde releasers and essential oils. Microbicides were tested as aqueous solutions of the active compounds and in complex formulations with sequestrants and ionic/non-ionic surfactants to mimic their real world use as hard-surface disinfectants (for BAC, chlorhexidine, DDAC, DMDM hydantoin, PHMB and thymol), and

laundry detergents (for BIT and triclosan). The reversibility of any induced susceptibility changes was also investigated to ascertain the stability of adaptation.

Reductions in bacterial susceptibility to an antimicrobial agent can be influenced by several factors related to the antimicrobial or the microorganism. Bacterial susceptibility may be affected by the structural integrity of the bacterial cell envelope and its ability to function as an effective permeability barrier (33-35). Innate bacterial non-susceptibility towards an antimicrobial agent may occur due to effective barrier components of the bacterial cell, such as an outer membrane in Gram-negative bacteria (36) or the spore coat in bacterial endospores (37). Changes in cell envelope permeability may therefore affect bacterial susceptibility which can include alterations in lipopolysaccharide expression and structure33, reduction in the number of outer membrane porins (23) and alterations in membrane fatty acid composition (38). The expression of efflux pumps has also been linked to decreases in microbicide susceptibility in bacteria, particularly towards membrane-active compounds such as biguanides (39) (CHX and PHMB) and quaternary ammonium compounds40 (BAC and DDAC in the current investigation). The increased expression of efflux pumps may therefore also provide a plausible explanation for some of the susceptibility changes observed in many of our bacterial isolates.
Reversible susceptibility changes to microbicides may result from temporary phenotypic adaptations in bacteria, such as the induction of stress responses that revert once the bacteria recover in an antimicrobial-free environment (41, 42). Equally, the development of microbicide insusceptibility may be attributable to the selection of insusceptible mutants, for instance mutations in FabI are reportedly render some bacteria insusceptible to triclosan (43, 44). However, the inherent stability of a particular mutation largely depends upon the overall fitness cost that it exerts on the host microorganism versus the competitive advantage that it provides in a particular environment (45). Hence, any mutation that renders a bacterium less susceptible

towards an antimicrobial agent may eventually be lost once the selective pressure is removed if the mutation results in a biologically significant reduction in the fitness of the microorganism (46).

Whilst previous studies have reported the induction of microbicide insusceptibility in bacteria, it should be noted that adapted bacterial isolates often remain susceptible to the microbicide at concentrations used in consumer products, and that true microbicide resistance is likely to be uncommon (25). In the current investigation, the only test bacterium that was refractory to a microbicide was *P. aeruginosa* to triclosan. This was apparent before microbicide exposure and has previously been attributed to the expression of efflux pumps 47. Interestingly this bacterium was comparatively susceptible to the triclosan formulation, illustrating marked differences in potency for the microbicide in aqueous solution compared to the formulated product.

Out of all the microbicides in unformulated form, BAC and triclosan induced the highest frequency of ≥4-fold increases in MIC with 6/9 bacterial isolates showing a reduction in susceptibility to both antimicrobials at this level. This was followed by PHMB (4 isolates) and CHX (3 isolates). Triclosan exposure resulted in the highest frequency of ≥4-fold increases in MBC (6 isolates) followed by PHMB (5 isolates), DDAC and BAC (3 isolates), then CHX (2 isolates) and DMDM hydantoin (1 isolate). In terms of the susceptibility of bacteria when grown as biofilms, PHMB adaptation resulted in the highest number of isolates showing ≥4-fold increases in MBEC (5 isolates) followed by triclosan and DDAC (4 isolates each) then BAC and CHX (2 isolates).

With respect to the formulated microbicides, BAC induced the highest number of ≥4-fold increases in MIC (4 isolates) followed by triclosan (3 isolates) and thymol (1 isolate). DMDM

hydantoin, thymol and PHMB containing formulations induced the largest number of ≥4-fold increases in MBC (3 isolates each) followed by BAC and DDAC (2 isolates each). Exposure to the DDAC containing formulations resulted in the highest numbers of bacterial isolates exhibiting a ≥4-fold increase in MBEC (4 isolates), followed by BAC and DMDM hydantoin (3 isolates) then PHMB, thymol and triclosan formulations (2 isolates).

Whilst the current investigation demonstrates that induced reductions in susceptibility towards both microbicides and microbicide-containing formulations may occur, a substantially higher number of bacterial isolates underwent ≥4-fold increases in MIC, MBC or MBEC when exposed to microbicides in aqueous solution, in comparison to those in formulation. The only exception to this was thymol, for which changes in susceptibility were more frequent in bacteria exposed to the compound in formulation. Thymol is poorly soluble in water and formulation may therefore have substantially improved solubility, increasing bacterial exposure and thus selectivity. Furthermore, since incorporating microbicides into formulations frequently enhanced antimicrobial potency, the formulated microbicides often maintained higher antimicrobial activity in comparison to microbicides in aqueous solution, even after repeated exposure. The incorporation of non-ionic surfactants and sequestrants into microbicide-containing formulations therefore appears to increase antimicrobial potency as well as mitigating the development of antimicrobial insusceptibility both in terms of frequency and magnitude of susceptibility change. Since excipients can interact with different cellular targets to the accompanying microbicide, formulations may have a cumulative antimicrobial effect which would require multiple further physiological adaptations to render the microorganism insusceptible.

Alcohol ethoxylates are a major class of non-ionic surfactants which are often used in household detergents, cleaners and personal care products and have previously shown bacteriostatic effects due to their direct impact on the bacterial cell membrane leading to the leakage of cytoplasmic components, indicating an increase in membrane permeability (48). An increase in membrane permeability would allow microbicides to more readily transverse the cytoplasmic membrane increasing their access to intracellular target sites. Therefore combining microbicides and alcohol ethoxylates in formulation may enhance overall antimicrobial potency, when compared to the pure active. Sodium tripolyphosphate, a chelating agent commonly used in domestic detergents, has previously shown antibacterial activity against several bacteria often found as food contaminants (49). Since sodium tripolyphosphate is a chelating agent it is plausible, as with other chelators such as EDTA, which this antibacterial activity occurs by disruption of the bacterial cell envelope through the sequestration of stabilising divalent cations. Such cations normally link bacterial lipopolysaccharides to the outer membrane and interference with this process can destabilise the outer membrane in Gram negative bacteria, impairing barrier function (50-52). Furthermore, strong chelating agents may inhibit bacterial growth by sequestering trace minerals required for bacterial metabolism (51, 53).

Essential oils such as thymol are often incorporated into antimicrobial formulation due to their inhibitory effects on bacterial growth. The antimicrobial activity of essential oils reportedly occurs through interaction with the bacterial cytoplasmic membrane, resulting in increased cell permeability and the disruption of energy generation (54, 55). Compensatory adaptations may occur, but whether these would result in outcome-changing effects during deployment depends on the extent of any susceptibility decreases, the concentration used in the product and the

antimicrobial potency of the formulation (i.e. the active compound and excipients in combination).

CONCLUSION

With the ultimate aim of developing realistic approaches to risk assessment, we observed that repeated exposure of 9 bacteria to 8 microbicides in aqueous solution or within complex formulations with sequestrants and ionic/non-ionic surfactants, induced reductions in bacterial susceptibility in a highly selective laboratory exposure system. Susceptibility changes varied in reversibility, possibly reflecting a range of underlying mechanisms including temporary phenotypic adaptation, such as the induction of stress responses or the selection of stable mutations. Importantly, the formulation of microbicides markedly increased overall antimicrobial potency for the test microbicides against the majority of the bacteria, as well as reducing the frequency and magnitude of susceptibility changes. Whilst it remains unclear how observations based on the *in vitro* exposure of bacteria to microbicides can be extrapolated to their use in the real world, understanding the potential selectivity of microbicide-containing formulations is likely to better served by testing formulations as well as actives aqueous solutions. This highlights the need to conduct risk assessments of induced microbicide susceptibility changes using conditions that more accurately reflect their deployment.

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TRANSPARENCY DECLARATION

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

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Table 1. Bacterial susceptibility towards benzalkonium chloride in planktonic and biofilm growth modes before, during and after repeated exposure to benzalkonium chloride in aqueous solution or in formulation

	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	0.1	**3.9**	**2.0**	0.5	**2.0**	**2.0**	2.0	**15.6**	**7.8**	2.0	**7.8**	**7.8**	2.6 (1)	**31.3**	**15.6**	3.9	**125**	7.8
E. coli†	4.6 (1)	**31.3**	**31.3**	3.9	**31.3**	**31.3**	7.2 (2)	**41.7 (16)**	**62.5**	7.8	**31.3**	**62.5**	31.3	31.3	62.5	31.3	62.5	62.5
E. faecalis†	2.0	**7.8**	3.9	2.0	3.9	3.9	3.3 (1)	7.8	7.8	3.9	7.8	7.8	6.5 (1)	**31.3**	7.8	6.7 (2)	**46.9 (17)**	**46.9 (17)**
P. aeruginosa†	14.3 (2)	**62.5**	**62.5**	15.6	**62.5**	**125**	23.4 (9)	**125**	**125**	31.3	62.5	**250**	125	250	**500**	62.5	**250**	**500**
*M. osloensis**	3.9	2.0	na	1.0	1.0	na	7.8	15.6	na	2.0	2.0	na	7.8	na	na	7.8	2.0	na
*A. baumanii**	2.0	**62.5**	**31.3**	3.9	**31.3**	**31.3**	93.8 (34)	250	125	62.5	62.5	125	125	250	125	125	125	93.8 (34)
*P. putida**	15.6	**62.5**	31.3	15.6	15.6	na	125	125	62.5	62.5	31.3	na	125	na	62.5	125	31.3	na
*C. sakazakii**	62.5	52.1 (16)	na	31.3	31.3	na	125	125	na	31.3	31.3	na	31.3	na	na	31.3	62.5	na
*E. coli**	18.4 (7)	52.1 (16)	na	15.6	31.3	na	62.5	125	na	31.3	31.3	na	62.5	na	na	62.5	62.5	na

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration.
Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *, drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). Organisms that underwent a ≥4-fold increase in MIC, MBC or MBEC (as indicated by bold text) were passaged a further 14 times in the absence of microbicide. na, bacteria that did not undergo a ≥4-fold change and were not assessed for reversibility. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses. In controls were bacteria were tested against formulations without microbicide, all bacteria were non-susceptible to in-use concentrations.

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550 **Table 2.** Bacterial susceptibility towards benzisothiozolinone in planktonic and biofilm growth modes before, during and after repeated exposure to
551 benzisothiozolinone in aqueous solution or in formulation

Bacterium	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	7.8	15.6	na	1.0	2.0	na	31.3	62.5	na	15.6	15.6	na	62.5	62.5	na	31.3	62.5	na
E. coli†	15.6	15.6	na	7.8	7.8	na	31.3	62.5	na	31.3	31.3	na	250	187.5 (68)	na	125	125	na
E. faecalis†	7.8	15.6	na	0.5	1.0	na	7.8	7.8	na	0.5	1.0	na	250	41.7 (16)	na	125	125	na
P. aeruginosa†	125	250	na	15.6	31.3	na	250	500	na	62.5	125	na	500	500	na	125+	125+	na
*M. osloensis**	1.0	1.0	na	0.5	0.5	na	1.0	1.0	na	0.5	0.5	na	2.0	2.0	na	0.5	1.0	na
*A. baumanii**	31.3	31.3	na	7.8	15.6	na	31.3	62.5	na	31.3	62.5	na	250	250	na	62.5	125	na
*P. putida**	15.6	31.3	na	31.3	31.3	na	62.5	62.5	na	31.3	62.5	na	250	250	na	62.5	125	na
*C. sakazakii**	7.8	7.8	na	7.8	7.8	na	31.3	31.3	na	31.3	31.3	na	250	500	na	62.5	125	na
*E. coli**	15.6	31.3	na	15.6	15.6	na	62.5	62.5	na	15.6	31.3	na	250	187.5	na	125	125	na

552 See footnote in Table 1

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556 **Table 3.** Bacterial susceptibility towards chlorhexidine in planktonic and biofilm growth modes before, during and after repeated exposure to chlorhexidine
557 in aqueous solution or in formulation

	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	1.7 (1)	**7.8**	3.9	2.0	2.0	na	5.2 (2)	**46.9 (17)**	**31.3**	7.8	7.8	na	13 (4)	31.3	31.3	7.8	15.6	na
E. coli†	2.4 (1)	**11.7 (4)**	7.9	2.0	3.9	na	9.8 (5)	**62.5**	**31.3**	15.6	31.3	na	52.1 (16)	62.5	31.3	62.5	31.3	na
E. faecalis†	3.9	7.8	15.6	3.9	7.8	na	14.3 (3)	31.3	31.3	7.8	15.6	na	31.3	**125**	62.5	31.3	62.5	na
P. aeruginosa†	7.8	**31.3**	**31.3**	7.8	15.6	na	68.8 (34)	**250**	125	125	125	na	250	125	125	250	125	na
*M. osloensis**	3.9	2.0	2.0	1.0	1.0	na	31.3	15.6	3.9	1.0	1.0	na	31.3	**125**	15.6	15.6	31.3	na
*A. baumanii**	7.8	7.8	na	3.9	7.8	na	125	62.5	na	15.6	31.3	na	125	125	na	125	31.3	na
*P. putida**	7.8	7.8	na	4.6 (2)	3.9	na	93.8 (34)	62.5	na	7.8	7.8	na	62.5	125	na	62.5	62.5	na
*C. sakazakii**	7.8	7.8	na	3.9	3.9	na	62.5	125	na	7.8	15.6	na	62.5	125	na	31.3	10.4 (4)	na
*E. coli**	7.8	10.4 (4)	15.6	3.9	3.9	na	46.8 (17)	125	125	7.8	15.6	na	125	125	125	62.5	23.4 (9)	na

558 See footnote in Table 1

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564 **Table 4.** Bacterial susceptibility towards didecyldimethyl ammonium chloride in planktonic and biofilm growth modes before, during and after repeated
565 exposure to didecyldimethyl ammonium chloride in aqueous solution or in formulation

	MIC						**MBC**						**MBEC**					
	UF			**F**			**UF**			**F**			**UF**			**F**		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	
S. aureus†	0.5	1.0	1.0	0.5	0.5	0.5	2.0	3.9	3.9	2.0	0.5	0.5	3.9	**31.3**	**31.3**	3.9	62.5	62.5
E. coli†	7.8	11.7 (4)	7.8	3.9	7.8	3.9	3.9	11.7 (4)	15.6	3.9	7.8	3.9	31.3	**125**	15.6	7.8	36.5 (13)	15.6
E. faecalis†	1.0	2.0	2.0	2.0	2.0	2.0	1.0	2.0	2.0	2.0	3.9	3.9	2.0	**125**	**31.3**	2.0	104.2 (32)	62.5
P. aeruginosa†	14.3 (2)	31.3	15.6	15.6	31.3	15.6	31.3	**125**	31.3	31.3	**125**	31.3	125	125	250	62.5	125	62.5
*M. osloensis**	1.0	1.0	1.0	1.0	1.0	na	1.4 (0.5)	3.9	2.0	2.0	2	na	2.0	3.9	3.9	2.0	2.0	na
*A. baumanii**	15.6	31.3	15.6	3.9	7.8	na	15.6	**62.5**	31.3	62.5	62.5	na	62.5	125	31.3	62.5	62.5	na
*P. putida**	47.4 (17)	31.3	na	4.6(1)	3.9	na	62.5	41.7 (17)	na	31.3	62.5	na	62.5	62.5	na	62.5	62.5	na
*C. sakazakii**	7.2 (2)	15.6	15.6	7.8	15.6	na	15.6	31.3	31.3	7.8	15.6	na	31.3	62.5	62.5	15.6	31.3	na
*E. coli**	4.6 (2)	15.6	15.6	3.9	7.8	3.9	10.4 (4)	**41.7 (17)**	31.3	3.9	**15.6**	7.8	15.6	**62.5**	31.3	15.6	62.5	23.5 (9)

566 See footnote in Table 1

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Table 5. Bacterial susceptibility towards Glydant (DMDM-hydantoin) in planktonic and biofilm growth modes before, during and after repeated exposure to Glydant (DMDM-hydantoin) in aqueous solution or in formulation.

	MIC						**MBC**						**MBEC**					
	UF			**F**			**UF**			**F**			**UF**			**F**		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	187.5	187.5	na	187.5	187.5	na	375	482 (183)	na	375	375	na	3000	3000	na	1500	3000	na
E. coli†	375	375	na	375	375	375	1500	1500	na	375	750	375	6000	6000	na	1500	**6000**	1500
E. faecalis†	187.5	187.5	na	187.5	187.5	na	1500	1500	na	1500	750	na	3000	3000	na	3000	6000	na
P. aeruginosa†	187.5	187.5	na	187.5	187.5	na	6000	6000	na	1500	1500	na	6000	6000	na	6000	12000	na
*M. osloensis**	375	375	na	46.9	62.5	na	325	375	na	187.5	187.5	na	750	1500	na	750	1500	na
*A. baumanii**	375	325	na	187.5	187.5	na	750	750	na	375	375	na	6000	6000	na	6000	6000	na
*P. putida**	375	375	na	375	375	na	750	750	na	750	375	na	6000	6000	na	3000	6000	na
*C. sakazakii**	375	375	na	187.5	187.5	375	3000	3000	na	375	750	375	6000	6000	na	1500	**6000**	1500
*E. coli**	187.5	466 (219)	187.5	187.5	375	187.5	375	**1500**	375	375	750	375	6000	6000	6000	1500	**12000**	1500

See footnote in Table 1

Table 6. Bacterial susceptibility towards PHMB in planktonic and biofilm growth modes before, during and after repeated exposure to PHMB in aqueous solution or in formulation

	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	3.9	**23.5 (9)**	**15.6**	3.9	3.9	3.9	3.9	**125**	**15.6**	3.9	**15.6**	7.8	15.6	**125**	15.6	15.6	**125**	31.3
E. coli†	15 (10)	31.3	15.6	7.8	15.6	na	15 (10)	**62.5**	**62.5**	15.6	31.3	na	62.5	62.5	62.5	62.5	31.3	na
E. faecalis†	7.8	**31.3**	15.6	5.9(1)	15.6	7.8	7.8	**125**	15.6	7.8	**31.3**	7.8	14.3 (3)	**125**	31.3	15.6	**125**	31.3
P. aeruginosa†	22.8 (15)	31.3	62.5	15.6	15.6	15.6	22.8 (15)	**125**	**125**	31.3	**125**	31.3	250	250	250	250	62.5	62.5
*M. osloensis**	7.8	**31.3**	3.9	1.0	1.0	na	62.5	31.3	31.3	7.8	7.8	na	62.5	62.5	31.3	31.3	62.5	na
*A. baumanii**	7.8	**31.3**	7.8	9.1 (3)	15.6	na	62.5	125	62.5	31.3	62.5	na	62.5	**250**	62.5	62.5	125	na
*P. putida**	28.9 (8)	31.3	na	15.6	15.6	na	62.5	62.5	na	31.3	62.5	na	125	125	na	125	125	na
*C. sakazakii**	7.8	15.6	15.6	31.2	15.6	na	104 (32)	125	125	15.6	31.3	na	62.5	**250**	125	62.5	125	na
*E. coli**	7.8	7.8	31.3	7.8	15.6	na	15.6	**250**	31.3	15.6	31.3	na	62.5	**250**	31.3	62.5	31.3	na

See footnote in Table 1

Table 7. Bacterial susceptibility towards thymol in planktonic and biofilm growth modes before, during and after repeated exposure to thymol in aqueous solution or in formulation

	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	187.5	187.5	na	187.5	187.5	na	375	375	na	375	750	na	416 (160)	375	na	375	750	na
E. coli†	1500	1500	na	187.5	375	375	1500	1500	na	375	**1500**	750	1500	1500	na	375	**3000**	1500
E. faecalis†	375	750	na	187.5	375	na	750	750	na	375	750	na	750	750	na	750	1500	na
P. aeruginosa†	3000	3000	na	1500	3000	na	6000	3000	na	3000	6000	na	6000	6000	na	6000	12000	na
*M. osloensis**	750	750	na	187.5	375	na	750	750	na	187.5	375	na	3000	1500	na	3000	375	na
*A. baumanii**	750	750	na	375	375	375	1500	3000	na	750	**6000**	**3000**	6000	6000	na	6000	6000	6000
*P. putida**	750	750	na	375	**3000**	375	1500	3000	na	1500	**6000**	3000	6000	6000	na	6000	6000	12000
*C. sakazakii**	750	750	na	375	375	na	2250 (822)	3000	na	375	750	na	6000	6000	na	3000	750	na
*E. coli**	665 (190)	750	na	187.5	375	na	3000	3000	na	375	750	na	6000	6000	na	750	**3000**	1500

See footnote in Table 1

Table 8. Bacterial susceptibility towards triclosan in planktonic and biofilm growth modes before, during and after repeated exposure to triclosan in aqueous solution or in formulation

Bacterium	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	0.2	**62.5**	**31.3**	0.1	0.1	0.1	3.9	**62.5**	**62.5**	0.1	0.1	0.1	65.1	125	125	2.0	**7.8**	2.0
E. coli†	2.0	**62.5**	**62.5**	0.1	**2.0**	**3.9**	2.0	**125**	**125**	7.8	7.8	3.9	125	**500**	125	62.5	15.6	15.6
E. faecalis†	62.5	62.5	62.5	0.1	0.1	0.1	62.5	125	125	0.1	0.1	0.1	15.6	**125**	**125**	2.0	**7.8**	2.0
P. aeruginosa†	ns	ns	ns	7.8	**62.5**	7.8	ns	ns	ns	62.5	62.5	7.8	ns	ns	ns	62.5	62.5	7.8
*M. osloensis**	1.0	**15.6**	**7.8**	1.0	1.0	na	7.8	**31.3**	**31.3**	3.9	3.9	na	125	125	125	3.9	3.9	na
*A. baumanii**	125	125	125	2.0	2.0	na	125	250	125	31.6	15.6	na	125	250	125	62.5	15.6	na
*P. putida**	15.6	**62.5**	**62.5**	1.0	2.0	na	62.5	125	125	15.6	15.6	na	125	250	500	62.5	15.6	na
*C. sakazakii**	7.8	**500**	**188**	2.0	2.0	na	7.8	**1000**	**250**	31.3	31.3	na	1.3 (0.5)	**125**	**125**	62.5	31.3	na
*E. coli**	1.0	**125**	**62.5**	0.1	**2.0**	**3.9**	2.0	**250**	**125**	15.6	15.6	15.6	125	**500**	125	62.5	15.6	15.6

See footnote in Table 1. ns, not susceptible (MBC/MIC/MBEC >1000 mg/L)