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1 **Use of a small-scale, portable test chamber for determining the**  
2 **bactericidal efficacy of aerosolized glycol formulations**

3

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20

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22

23

24 **Significance and Impact of the study:** There is an increased interest in  
25 developing effective microbicidal aerosolised formulations. The development of  
26 a small in-house test chamber allowed the measurement of the microbicidal  
27 efficacy of an aerosolised glycol/ethanol formulation at a low cost. We showed  
28 that a glycol/ethanol aerosolised formulation caused extensive structural  
29 damage in Gram-negative and -positive bacteria resulting in a 3 log<sub>10</sub> reduction  
30 in viability.

31

### 32 **Abstract**

33 This study aimed to understand the efficacy and mechanisms of action of an  
34 aerosolised glycol-ethanol formulations against bacteria. We validated a small  
35 scale in-house test chamber to determine the microbicidal efficacy of four  
36 aerosolized formulations combining dipropylene glycol and ethanol against  
37 *Staphylococcus aureus* and *Escherichia coli* embedded in alginate. The  
38 aerosolised glycol/ethanol formulation decreased bacterial viability by 3 Log<sub>10</sub>  
39 and was more efficacious than an ethanol only control formulation. Electron  
40 microscopic examination indicated extensive structural damage in both bacteria,  
41 and membrane damage was confirmed with potassium release in *S. aureus*  
42 and DNA release in *E. coli*. The development of a small test chamber facilitated  
43 the measurement of the microbicidal efficacy and experiments to understand  
44 the mechanism of action of an aerosolised microbicidal formulation.

45

46

47 **Introduction**

48 Pathogens discharged into the air may settle on environmental surfaces, which  
49 could then become secondary vehicles for the spread of infectious agents  
50 indoors occurring at the air-surface-air nexus (Gralton *et al.*, 2011; Ijaz *et al.*,  
51 2016). Considering the concern about the potential spread of microorganisms  
52 indoors and the limited techniques for testing new formulations, Sattar and  
53 colleagues (2016) designed a protocol to study survival and inactivation of  
54 human pathogens in indoor air. These authors tested the efficacy of a  
55 microbicidal formulation containing 4% dipropylene glycol (DPG) and 35%  
56 ethanol distributed within a 24.3 m<sup>3</sup> aerobiology chamber using an air purifier  
57 with a newly-designed fogger (Sattar *et al.* unpublished data).

58 The study of human pathogens aerosols requires the ability to produce them  
59 experimentally in appropriate droplet size and sample them safely for analysis  
60 over a predetermined time periods (Sattar and Ijaz, 1987). Since the recovery  
61 of aerosolised bacterial inocula from large-scale microbicide experiments  
62 presents several technical challenges, the immobilization of bacteria in an  
63 alginate support may provide an appropriate alternative (Shackelford *et al.*,  
64 2006). The alginate matrix provides sufficiently large pores to allow the easy  
65 penetration of microbicide and may enable testing of microbicidal efficacy  
66 without the confounding effects of bacterial aerosol-induced cell injury or drying  
67 (Tattawasart *et al.*, 2000a; Shackelford *et al.*, 2006) or aerosolising bacteria  
68 with the negative effects of aerosolising process on bacterial viability (O'Jeil *et al.*  
69 *et al.*, 2013). In addition, the use of an alginate matrix allows recovering bacteria  
70 in high number for the study of mechanisms of action (Tattawasart *et al.*, 2000a;  
71 Shackelford *et al.*, 2006). Glycols such as propylene glycol (PG) and triethylene

72 glycol (TEG) are greatly used in formulations, generally as solvent, extractant  
73 and on occasions as preservatives at concentrations 15-30% (Rowe *et al.*,  
74 2009). The microbicidal activity of glycols has however not been widely  
75 reported.

76 The aim of this study was to measure the bactericidal efficacy of several  
77 aerosolised glycol-based formulations and their mechanisms of microbicidal  
78 action using a small scale aerosolisation test chamber.

79

## 80 **Results and discussion**

81

82 There is a great interest in gaseous and air decontamination technologies  
83 notably in healthcare settings (Davies *et al.*, 2011). The use of highly reactive  
84 chemistry such as vaporized hydrogen peroxide has been successful in  
85 controlling pathogen outbreaks in healthcare environments (Ray *et al.*, 2010;  
86 Goyal *et al.*, 2014). Aerosolised chlorine-based microbicides have also been  
87 explored against *Staphylococcus aureus* with various degrees of efficacy  
88 (Thorn *et al.*, 2015). However, these highly reactive chemistries are toxic and  
89 the room to be decontaminated needs to be vacated and sealed during the  
90 gaseous process. In addition, testing the efficacy of aerosolised formulations is  
91 expensive and require the use of specifically designed aerobiology test  
92 chambers (Sattar *et al.*, 2016). Here, we successfully developed a small scale  
93 test chamber to study the efficacy and mechanisms of action of aerosolised  
94 formulations. The combination of the chamber with an immobilised bacterial  
95 inoculum allowed the recovery of a high bacterial inoculum facilitating the study  
96 of the formulations' mechanisms of action.

97

98 *Effect of liquid formulations on bacterial growth*

99 We are not aware of any scientific publications describing of the microbicidal  
100 activity of dipropylene glycol. Our results show that the test formulations,  
101 including dipropylene glycol only (6.67%) inhibited the growth of the test  
102 bacteria. All liquid formulations affected bacterial growth in comparison to the  
103 TSB positive growth control (Figures 2 and 3). The formulations containing  
104 glycol, or ethanol or a combination of glycol and ethanol had a more  
105 pronounced effect inhibiting completely the growth of both bacteria comparing  
106 to the blank formulation that affected bacterial growth rate and final OD value  
107 (Figures 2 and 3).

108

109 *Bactericidal efficacy of aerosolised formulations*

110 Percentage recoveries of bacteria embedded in the alginate were 90.8% and  
111 91.63% for *E. coli* and *S. aureus*, respectively. The use of the fogger  
112 formulations containing glycol in our test chamber (10 sec aerosol exposure,  
113 40-60% RH) produced a reduction in bacterial viability. However, the complete  
114 formulation (Glycol + Ethanol) was significantly (ANOVA;  $P < 0.001$ ) more  
115 efficacious with a reduction of  $3.20 \pm 0.13 \text{ Log}_{10}$  for *S. aureus aureus* and  $3.19$   
116  $\pm 0.39 \text{ Log}_{10} \text{ cfu ml}^{-1}$  for *E. coli* compared to  $1.54 \pm 0.31$  and  $1.41 \pm 0.15$  with  
117 each bacterium respectively (Table 3). There were no statistically significant  
118 differences (ANOVA;  $P > 0.1$ ) between the efficacy of the glycol only, the ethanol  
119 only formulations and the blank formulation (Table 3). The use of chlorine-  
120 based aerosols (with 20 min aerosol exposure, 10 min resting time, and 50%RH)  
121 produced 1-5  $\log_{10}$  reduction in *S. aureus* concentration on stainless steel

122 surfaces depending on the initial chlorine solution (sodium hypochlorite,  
123 chlorine dioxide or electrochemically activated solution) (Thorn et al., 2013).  
124 Gaseous chlorine (250 mg l<sup>-1</sup>) has been used *in situ* to decontaminate indoor  
125 air pathogens and contributed to a reduction <1500 cfu m<sup>3</sup> in bacteria and of  
126 <1000 cfu m<sup>3</sup> in fungi (Hsu et al., 2015).

127 The bactericidal activity of other glycols has been reported. Berry (1944)  
128 reported MIC ethylene glycol monophenyl ether of 0.8% v/v against *S. aureus*  
129 and 0.5 % v/v against *E. coli* while a number of chloro-and methyl-substituted  
130 aryl ethers of glycerol, propylene glycol and trimethylene glycol were shown to  
131 have some activity against bacteria, fungi and yeast (Berger et al., 1953).  
132 Propylene glycol has been used as a solvent in combination with phenol with  
133 report of some bactericidal activity (Baker and Twort, 1941). It is however clear  
134 that RH played an important role in the activity of the formulation (Baker and  
135 Twort, 1941). Chirife and colleagues (1983) studied the microbicidal activity of  
136 polyethylene glycol 400 and suggested that the bactericidal activity observed  
137 at 35°C was a combination of lowering water activity and a direct effect of  
138 bacterial cells demonstrated by cell clumping. Recently ethylene glycol  
139 bactericidal activity was reported against *E. coli* with MIC and MBC values of  
140 18 and 24 % v/v (Moghayedi et al. 2017).

141

#### 142 *Mechanisms of bactericidal action of aerosolised glycol formulations*

143 The mechanisms of action of the aerosolised formulations were explored. It was  
144 hypothesized that the bactericidal activity observed resulted from membrane  
145 damage. Using the FEI Quanta 200F software (Eindhoven, The Netherlands),  
146 direct SEM examination showed evidence of structural damage for both *S.*

147 *aureus* and *E. coli* with all the formulations tested (Figures 4 and 5). Ninety  
148 percent of the bacterial population was damaged following exposure to the  
149 control formulations (Table 3). Such level of inactivation contributes to create a  
150 substantial artefact as demonstrated from the SEM images and OMP analysis.  
151 Loss of bacterial viability during sample preparation prior to testing has been  
152 well reported. The decrease in bacterial concentration depends on the material  
153 used (Best et al., 1988; Thorn et al., 2013) and the type of bacteria; some recent  
154 European efficacy test protocol such as the EN14776 recommends the use of  
155 glycerol with the Gram-negative bacterial inoculum to protect test inocula from  
156 dehydration. Here, the use of the alginate limited bacterial loss and contributed  
157 to the use of a highly reproducible test inoculum concentration, as described by  
158 Shackelford and colleagues (2006).

159 It is clear that the aerosol of the blank formulation by itself caused membrane  
160 damage, with indication that the Gram-negative was more affected with release  
161 of DNA, although not to the OMP. Although the extent of damage could not be  
162 quantified, the combined formulations seems to disrupt structural integrity of *S.*  
163 *aureus* more extensively when compared to the other formulations (Figure 4).  
164 The severity of damage caused by the combined formulation was more  
165 apparent in *E. coli* (Figure 5). Exposure to the glycol only or ethanol only  
166 formulation seems to 'smooth' the surface of *E. coli* while the blank formulation  
167 affected the bacterial surface but not to same extent as the combined  
168 formulation (Figure 5). SEM images indicated structural changes when bacteria  
169 were exposed to the formulations with the different formulations. Damage  
170 caused by the combined glycol and ethanol formulation seemed more severe  
171 in both Gram-negative and Gram-positive bacteria. A recent study showed



172 severe bacterial structural damage in *E. coli* exposed to 25% ethylene glycol  
173 (Moghayedi et al. 2017).

174 Potassium leakage measurements indicated that the full formulation may  
175 interact with the cytoplasmic membrane of the Gram-positive bacterium,  
176 releasing some potassium, but not larger cell components. Potassium release  
177 is the first indicator of membrane damage followed by larger cytoplasmic  
178 constituents (Maillard, 2002). Here, a small but statistically significant increase  
179 (ANOVA;  $P < 0.001$ ) in potassium leakage was observed when testing the glycol  
180 and ethanol (A), and glycol only formulations (B) against *S. aureus* compared  
181 to the blank formulation (D) (Table 4). No potassium was released from *E. coli*  
182 exposed to the aerosolised formulations (Table 4).

183 The release of DNA from bacteria following exposure to the aerosolised  
184 formulations was measured spectrophotometrically following exposure. The  
185 OD<sub>260</sub> reading allows for the calculation of the concentration of nucleic acid in  
186 the sample. An OD of 1 corresponds to ~50 µg ml<sup>-1</sup> for DNA. The OD<sub>260</sub>/OD<sub>280</sub>  
187 ratio reading provides an estimate of the purity of the nucleic acid. Pure  
188 preparations of DNA have OD<sub>260</sub>/OD<sub>280</sub> values of ≥1.8. There were more DNA  
189 in the *E. coli* samples than in the *S. aureus* ones (Table 5). Exposure to the  
190 aerosolised formulation contained glycols and ethanol or to the nebulised blank  
191 formulation resulted in very little DNA release, 4% and 6% of total estimated  
192 DNA in *S. aureus* and *E. coli* respectively.

193

194 The results of SDS-PAGE indicated no changes in OMP in *E. coli* after  
195 exposure to the blank and the formulation containing combined glycols and  
196 ethanol were detected by the technique. There was no apparent change in the

197 OMP profile following exposure to the blank or the combined glycol and ethanol  
198 formulation, although an increase in the band concentration of three specific  
199 OMP of 35 kD, 27 kD and 16 kD approximately was observed when bacteria  
200 were exposed to the aerosolised formulations (Figure 6).

201 In conclusion, the test chamber described in our study allowed the microbicidal  
202 evaluation of foggers containing glycol-based formulations and also  
203 nebulization of blank formulation against bacteria embedded in alginate matrix.  
204 The advantage of the small size chamber is the rapidity of the experiment and  
205 cost to set up (compared to the use of an aerobiology chamber). One limitation  
206 is that the aerosolised formulations need to be adapted to the small volume of  
207 our test chamber. The bactericidal efficacy of the combined aerosolised glycol  
208 and ethanol formulation could be partly attributed to damaging the cytoplasmic  
209 membrane of Gram-positive bacteria and to damaging the outer membrane of  
210 Gram-negative bacteria.

211

## 212 **Material and methods**

213

### 214 *Aerosolized Formulations*

215 Four formulations (Table 1) combining DPG and anhydrous ethanol provided  
216 by Reckitt Benckiser (One Philips Parkway, Montvale, NJ 07645. USA) were  
217 studied. Samples were stored at room temperature prior to fogging.

218

### 219 *Bacterial strains*

220 *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 11229)  
221 commonly used in standard efficacy test protocols were used as test bacteria.

222 Both strains were stored on Nutriprotect beads (Fisher Scientific,  
223 Loughborough, UK) at  $-80 \pm 1^{\circ}\text{C}$  and restricted to a maximum of 2 subcultures  
224 from the original freezer stock prior to any testing. Both strains were grown in  
225 tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) at  $37 \pm 1^{\circ}\text{C}$  for 16-24 hours.  
226 Test inoculum were prepared from harvesting an overnight TSB culture  
227 centrifuged at 5,000 g for 10 minutes and re-suspended in phosphate buffer  
228 saline (PBS) (Fisher Scientific, Loughborough, UK).

229

### 230 *Sodium alginate test inoculum preparation*

231 The effects of aerosolised formulations were analysed against vegetative  
232 bacteria embedded in sodium alginate. The test protocol was adapted from the  
233 procedure described in Shackelford *et al.* (2006). A 1 ml sample of bacterial  
234 suspension was added to a 1 ml cooled 3% (w/v) sodium alginate solution in  
235 deionised water and mixed by pipetting (BDH Chemicals Ltd, Poole, UK). The  
236 final concentration in the bacteria/alginate mixture was  $1-10 \times 10^9$  cfu ml<sup>-1</sup>.  
237 Aliquot of alginate/bacteria (0.2 ml) was dispensed onto the centre of a moulds  
238 holder system of M8 flat stainless still washers placed on top of stainless still  
239 coupons grade 2B of 0.22 mm X 0.22 mm (Goodfellows Cambridge Ltd.  
240 Huntington, UK). Both, flat washers and coupons were soaked with 5 %  
241 Decon90 (Decon Laboratories Limited, Hove, UK) in deionised water for 60 min,  
242 rinsed, dried and then autoclaved before use.

243 Loaded mould holder system was placed into individual wells of sterile 6 well  
244 plates (Corning® Costar®, Sigma-Aldrich UK) containing 10 ml of 2% calcium  
245 chloride solution (BDH chemicals, Poole, UK) and left for 5 min to form a gel as

246 the sodium ions are exchanged with calcium ions and the polymers become  
247 cross-linked (Waldman *et al.*, 1998).

248 To recover the bacteria from the alginate matrix, both control (no formulation  
249 added) and post formulations exposure, the gels were rinsed in five changes of  
250 10 ml of sterile distilled water and dissolved in 10 ml of McIlvaine's buffer (0.1  
251 mol l<sup>-1</sup> citric acid and 0.2 mol l<sup>-1</sup> disodium phosphate at pH 7.4; Fisher Scientific).  
252 Samples were taken from the dissolved gels, serially diluted in sterile PBS (pH  
253 7.4; Fisher Scientific) and CFU counts were performed using the Miles and  
254 Misra drop count method. The bacterial recovery and microbicidal efficacy (BE)  
255 was calculated as follows:  $BE = \log N_c - \log N_b$  where  $N_c$  and  $N_b$  represent the  
256 numbers of CFU ml<sup>-1</sup> in the control and biocide fogger formulations, respectively.  
257

#### 258 *In-house test chamber conditions*

259 A small portable aerosolisation test chamber was modified from the test  
260 chamber described by O'Jeil and colleagues (2013). Fogger release conditions  
261 were adapted to the size of the chamber consisted of a 23 cm stainless steel  
262 tube connected at the other end to an Andersen cascade impactor (Westech  
263 Instrument Services Ltd, Henlow, UK). A constant low flow rate measured by a  
264 Copley Scientific DFM2000 (Nottingham, UK) flow meter was generated  
265 through a vacuum pump (Fisherbrand, Loughborough, UK) connected to the  
266 Andersen cascade impactor (Figure 1). Temperature and relative humidity  
267 conditions on surfaces were determined using a S154TH temperature and  
268 relative humidity probe. Testing parameters were established according to the  
269 chamber dimension size. Test operation conditions (Table 2) were set up to  
270 mimic a previous study performed with the aerobiology chamber (Sattar *et al.*,

271 2016). The procedure was carried out in a class-2 microbiological safety  
272 cabinet facility.

273 The blank formulation (Table 1) could not be delivered by fogger due to  
274 excessive foam release. Instead the blank formulation was nebulised using a  
275 nebuliser (Philips Respronic, Best, The Netherlands) connected to the 23 cm  
276 stainless steel tube of the rig. All test parameters were the same for all tested  
277 formulations.

278

#### 279 *Bactericidal activity of formulations in suspension*

280 Bacterial growth kinetics was determined using the Bioscreen C Microbial  
281 Analyser (Labsystems, Helsinki, Finland) for both microorganisms using the  
282 four formulations as solutions: glycol+ethanol (A), glycol (B), ethanol (C) and  
283 blank (D). Controls consisted of each bacterium growing in TSB. The Bioscreen  
284 was run for 24 h at 25°C and readings were taken using a wideband filter (420-  
285 580nm) every 15 min preceded by 10 s shaking.

286

#### 287 *Microbicide mechanisms of action*

288 Bacterial gross structural damage following exposure to aerosolised  
289 formulations was explored by scanning electron microscopic examination  
290 (SEM imaging) (Walkera *et al.*, 2003) using the FEI Quanta 200F (Eindhoven,  
291 The Netherlands). Loss of (cytoplasmic) membrane integrity was measured  
292 with potassium leakage according to Walsh *et al.* (2003). A number of controls  
293 were performed including boiled bacteria at 80°C for 20 min (maximum  
294 potassium release; positive control 1), boiled bacteria embedded in the matrix  
295 at 80°C for 20 min (effect of alginate on potassium release; positive control 2),

296 effect of nebulisation on the release of K<sup>+</sup> from embedded bacteria in the  
297 alginate (nebulisation control). Potassium release was measured by inductively  
298 coupled plasma mass spectrometry (Agilent 7900 ICP-MS). A five-point  
299 calibration (1, 0.1, 0.01 and 0.001 mg l<sup>-1</sup>, (Tune) and calibration blank (CAL))  
300 was run and an internal standard (IS) was used throughout the analysis. All  
301 standards (Tune/Calibration and IS) are certified reference standards from  
302 Agilent. Samples were run in duplicate with blanks in between different samples  
303 to ensure there was no carryover of K<sup>+</sup>.

304 To evidence gross membrane damage, DNA release was measured by UV  
305 spectrometry following exposure to the four formulations. Following exposure  
306 to aerosolised formulation and dissolution of the alginate, samples were added  
307 to a 1 cm path-length cuvette and OD recorded 260 nm and at 280 nm. Samples  
308 with an OD of 1 at 260 nm contain approximately 50 µg ml<sup>-1</sup> double-stranded  
309 DNA (Nicklas and Buel, 2003).

310 Positive controls consisted in DNA measured spectrophotometrically of boiled  
311 bacteria at 80°C during 20 min.

312 Damage to the Gram-negative outer membrane proteins (OMP) after exposure  
313 to the different formulations were examined using SDS-PAGE according to  
314 Tattawasart and colleagues (2000b) with the silver staining performed  
315 according to Hitchcock and Brown (1983). ImageJ software (Schindelin *et al.*,  
316 2012) was used as a semi-quantitative tool to determine the amount of OMP  
317 observed.

318

319 *Statistical analysis*

320 All experiments were performed in triplicate unless otherwise stated. Bacterial  
321 reduction and effects of the possible mechanism of action of the formulations  
322 tested were compared by one-way ANOVA and Tukey tests with a 95%  
323 confidence level using Rstudio software (Version 1.1.383). Results were  
324 considered significant when  $P < 0.001$ .

325

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328

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332

### 333 **Conflict of interest**

334 JR Rubino and Ijaz MK are employees of Reckitt Benckiser.

335

### 336 **References**

337 Baker, A.H. and Twort, C.C. (1941) The effect of humidity of air on the  
338 disinfection capacity of mechanically atomized and heat-volatilized  
339 germicidal aerosols. *J Hyg* **41**, 117-130.

340 Berger, F.M., Hubbard, C.V. and Ludwig, B.J. (1953) The antimicrobial action  
341 of certain glycerol ethers and related compounds. *Appl Microbiol* **1**, 146-149.

342 Berry, H. (1944) Antibacterial values of ethylene glycol monophenyl ether. *The*  
343 *Lancet* **247**, 175-176

344 Best, M., Sattar, S. A., Springthorpe, V.S., Kennedy, M.E. (1988) Comparative  
345 mycobactericidal efficacy of chemical disinfectants in suspension and  
346 carrier tests. *Appl Environ Microbiol* **54**, 2856-2858.

347 Chirife, J., Herszage, L., Joseph, A., Bozzini, J.P., Leardini, N. and Kohn, E.S.  
348 (1983) In vitro antibacterial activity of concentrated polyethylene glycol 400  
349 solutions. *Antimicrob Agents Chemother* **24**, 409-412.

350 Davies, A., Pottage, T., Bennett, A. and Walker, J. (2011) Gaseous and air  
351 decontamination technologies for *Clostridium difficile* in the healthcare  
352 environment. *J Hosp Infect* **77**,199-203.

353 Gralton, J., Tovey, E., McLaws, M.L., Rawlinson, W.D. (2011) The role of  
354 particle size in pathogen transmission: a review. *J Infect* **62**, 1-13.

355 Goyal, S.M., Chander, Y., Yezli, S. and Otter, J.A. (2014) Evaluating the  
356 virucidal efficacy of hydrogen peroxide vapour. *J Hosp Infect* **86**, 255-259.

357 Hitchcock, P.J. and Brown, T.M. (1983) Morphological heterogeneity among  
358 *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide  
359 gels. *J Bacteriol* **154**, 269-277.

360 Hsu, C.-S., Lu, M.-C. and Huang, D.-J. (2015) Disinfection of indoor air  
361 microorganisms in stack room of university library using gaseous chlorine  
362 dioxide. *Environ Monitor Assess* **187**, DOI: 10.1007/s10661-014-4235-2

363 Ijaz MK, Zargar B, Wright KE, Rubino, J.R., and Sattar, S.A. Genetic aspects  
364 of the airborne spread of human pathogens indoors and emerging air  
365 decontamination technologies. *Am J Infect Control* 2016;**44**:S109-S120.

366 Maillard, J.-Y. (2002). Bacterial target sites for biocide action. *Symp Ser Soc*  
367 *Appl Microbiol Symposium Supplement* **92**, 16S–27S.



368 Moghayedi, M., Ahmadzadeh, H., Ghazvini, K. and Goharshadi, E.K. (2017)  
369 Neglected antibacterial activity of ethylene glycol as a common solvent.  
370 *Microb Pathogen* **107**, 457-46.

371 Nicklas, J.A. and Buel, E. (2003). Quantification of DNA in forensic samples.  
372 *Anal Bioanal Chem* **376**, 1160-1167.

373 Ojeil, M., Jermann, C., Holah, J., Denyer, S., and Maillard, J.-Y. (2013)  
374 Evaluation of antimicrobial surface activity with a newly developed in vitro  
375 efficacy test reflective of conditions found in UK hospitals. *J Hosp Infect* **85**,  
376 274-281.

377 Ray, A., Perez, F., Beltramini, A.M., Jakubowycz, M., Dimick, P., Jacobs, M.R.,  
378 Roman, K., Bonomo, R.A. and Salata, R.A. (2010) Use of vaporized  
379 hydrogen peroxide decontamination during an outbreak of multidrug-  
380 resistant *Acinetobacter baumannii* infection at a long-term acute care  
381 hospital. *Infect Control Hosp Epidemiol* **31**,1236–1241

382 Rowe, R.C., Sheskey, P.J. and Quinn, M.E. (eds.)(2009). Handbook of  
383 Pharmaceutical Excipients, 6<sup>th</sup> edn. Pp. 592-594. Pharmaceutical Press:  
384 London

385 Sattar, S.A. and Ijaz, M.K. (1987). Spread of viral infections by aerosols. *Crit*  
386 *Rev Environ Control* **17**, 89-131.

387 Sattar, S.A., Kibbee, R.J., Zargar, B., Wright, K.E., Rubino, J.R., Ijaz, M.K.  
388 (2016) Decontamination of indoor air to reduce the risk of airborne  
389 infections: studies on survival and inactivation of airbornes pathogens  
390 using an aerobiology chamber. *Am J Infect Control* **44**, e177-e182.

391 Schindelin, J., Arganda-Carreras, I., Frise, E. et al. (2012), Fiji: an open-source  
392 platform for biological-image analysis. *Nature Meth* **9**, 676-682.

393 Shackelford, J.C.N., Hanlon, G. and Maillard, J.-Y. (2006) Use of a new alginate  
394 film test to study the bactericidal efficacy of the high-level disinfectant ortho-  
395 phthalaldehyde. *J Antimicrob Chemother* **57**, 335-338.

396 Tattawasart, U., Hannb, A.C., Maillard, J.-Y., Furra, J.R. and Russell, A.D.  
397 (2000a) Cytological changes in chlorhexidine-resistant isolates of  
398 *Pseudomonas stutzeri*. *J Antimicrob Chemother* **45**, 145-152.

399 Tattawasart, U., Maillard, J.-Y., Furr, J.R. and Russell, A.D. (2000b) Outer  
400 membrane changes in *Pseudomonas stutzeri* resistant to chlorhexidine  
401 diacetate and cetylpyridinium chloride. *Int J Antimicrob Agents* **16**, 233–238.

402 Thorn, R.M.S., Robinson G.M. and Reynolds, D.M. (2013) Comparative  
403 antimicrobial activities of aerosolized sodium hypochlorite, chlorine dioxide,  
404 and electrochemically activated solutions evaluated using a novel  
405 standardized assay. *Antimicrob Agents Chemother* **57**,2216-2225.

406 Walkera, M., Hobotb, J.A., Newmanb, G.R. and Bowlera, P.G. (2003).  
407 Scanning electron microscopic examination of bacterial immobilization in a  
408 carboxymethyl cellulose (AQUACELs) and alginate dressings. *Biomaterials*  
409 **24**, 883–890.

410 Walsh, S.E., Maillard, J-Y., Russell, A.D., Catrenich, C.E., Charbonneau, C.E.  
411 and Bartolo, R.G. (2003) Activity and mechanisms of action of selected  
412 biocidal agents on Gram-positive and Gram-negative bacteria. *J Appl*  
413 *Microbiol* **94**, 240-247.

414 Waldman, A.S., Schechinger, L., Govindarajoo, G., Nowick, J.S. and Pignolet,  
415 L.H. (1998) The alginate demonstration: polymers, food science, and ion  
416 exchange. *J Chem Educ* **75**, 1430-1431.

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421 **Table 1** Aerosolized formulations tested

Sample	Active	Alcohol	Concentration (%)
Full formulation (A)	Dipropylene glycol	Anhydrous ethanol	6.67% / 46.67%
Glycol formulation (B)	Dipropylene glycol	-	6.67%
Ethanol formulation (C)	-	Anhydrous ethanol	46.67%
Blank formulation (D)	-	-	0

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423 **Table 2** In-house chamber testing conditions

Parameter	Specification	Condition tested
Chamber dimension	Size	230 cm <sup>3</sup>
Test operation	Air rate (flow)	2 l min <sup>-1</sup>
	Soil load	-
	Fogger release	10 sec
	Relative humidity	40-60%
	Temperature	24°C
Glycol release	Concentration	0.18 g
Microorganisms tested	Bacterial concentration	1 x 10 <sup>9</sup> CFU ml <sup>-1</sup>

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425 **Table 3** Bactericidal efficacy of aerosolised formulations against *S. aureus* and *E. coli* with a flow of 2.0 l min<sup>-1</sup>.

Formulation	<i>S. aureus</i> (Log <sub>10</sub> ± SD)			<i>E. coli</i> (Log <sub>10</sub> ± SD)		
	T=0	T=10 sec		T=0	T=10 sec	
		Recovery	Reduction		Recovery	Reduction
Complete formulation- Glycol and Ethanol	9.93 ± 0.15	6.73 ± 0.04	3.20 ± 0.13	9.93 ± 0.05	6.74 ± 0.33	3.19 ± 0.39
Glycol only formulation	9.68 ± 0.00	8.12 ± 0.20	1.56 ± 0.20	9.68 ± 0.11	7.81 ± 0.18	1.87 ± 0.26
Ethanol only formulation	9.68 ± 0.00	7.90 ± 0.20	1.78 ± 0.20	9.68 ± 0.11	7.61 ± 0.44	2.07 ± 0.52
Blank formulation	9.38 ± 0.00	7.84 ± 0.31	1.54 ± 0.31	9.07 ± 0.00	7.66 ± 0.15	1.41 ± 0.15

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427 **Table 4** Potassium release from *S. aureus* and *E. coli* after exposure with  
 428 aerosolised or nebulised formulations.

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430 Potassium Release (ppm)

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SAMPLE (n=2)	<i>S. aureus</i>	<i>E. coli</i>
Positive control: bacteria only	100.99	26.75
Positive control: bacteria + matrix	48.51	12.36
Nebulisation control	0.57	0.14
Blank formulation	0.69	0.19
Complete formulation-Glycol and Ethanol	0.91	0.26
Glycol only formulation	0.87	0.19
Ethanol only formulation	0.49	0.18

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432 **Table 5** DNA release from *S. aureus* and *E. coli* following exposure to the aerosolised formulations.

SAMPLE (n=2)	OD <sub>260</sub>	OD <sub>280</sub>	OD <sub>260</sub> :O D <sub>280</sub>	Estimated DNA concentration (µg ml <sup>-1</sup> )
<b><i>S. aureus</i></b>				
Positive control (boiled bacteria)	0.50	0.20	2.5	25.0
Complete formulation-Glycol and Ethanol	0.02	0.02	1.00	1.0
Blank formulation	0.02	0.01	1.67	1.0
<b><i>E. coli</i></b>				
Positive control (boiled bacteria)	3.00	2.02	1.49	150
Complete formulation-Glycol and Ethanol	0.19	0.09	2.01	9.5
Blank formulation	0.13	0.07	1.87	6.5

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434 **Figure 1.** In-house aerosolised test chamber

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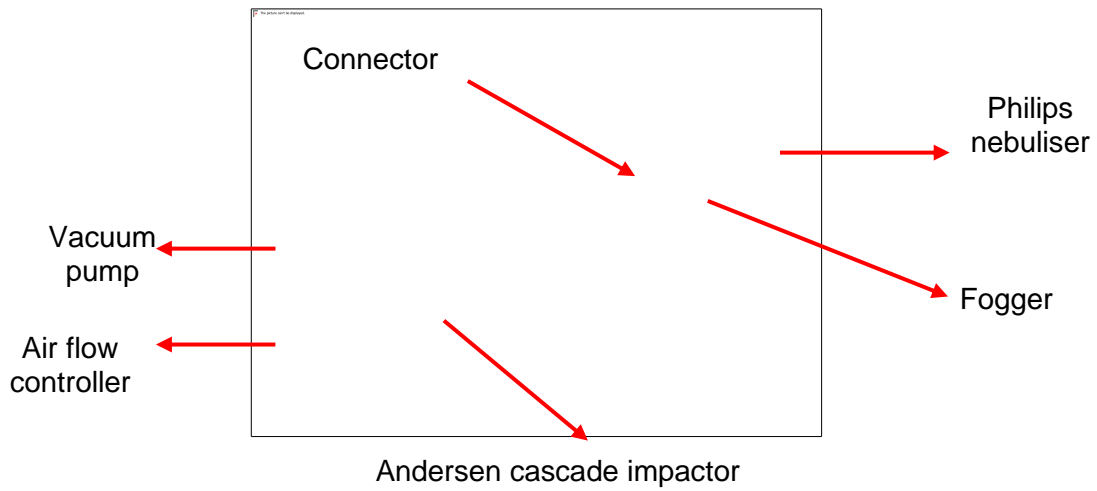
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443 **Figure 2** Effect of the tested formulations on the growth of *S. aureus*. (■)

444 Complete formulation-Glycol and Ethanol; (■): Glycol only formulation; (■)

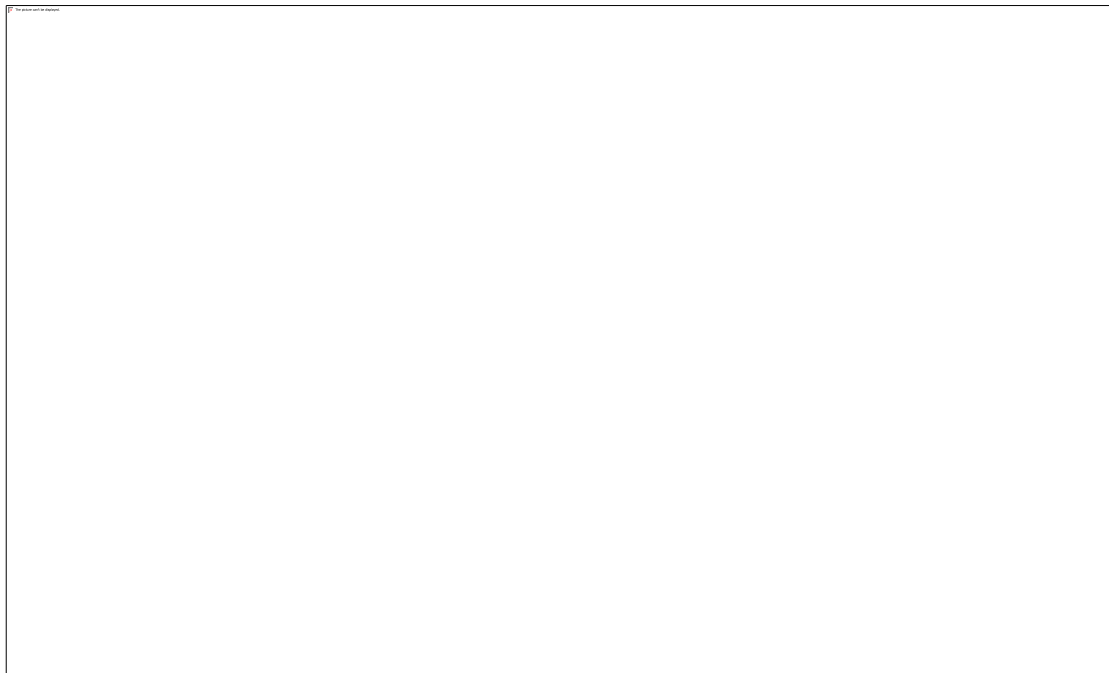
445 Ethanol only formulation; (■): Blank formulation and (■): growth control (TSB)

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450 **Figure 3** Effect of the tested formulations on the growth of *E. coli*. (■) Complete  
451 formulation-Glycol and Ethanol; (■): Glycol only formulation; (■) Ethanol only  
452 formulation; (■): Blank formulation and (■): growth control (TSB). (n=32)



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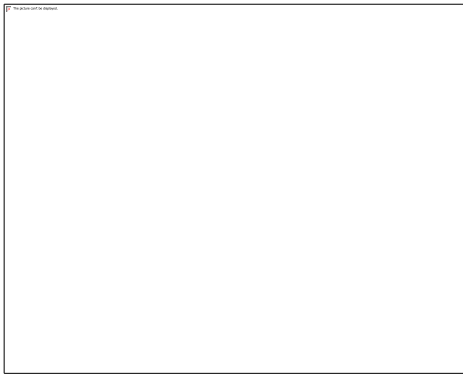
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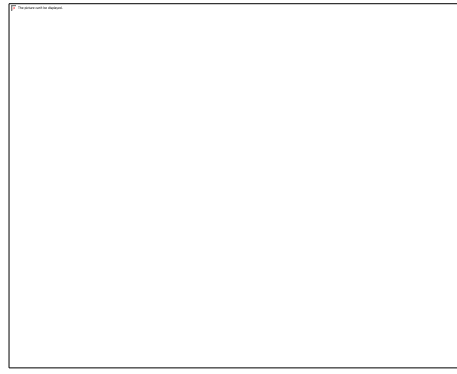
456 **Figure 4** SEM images of *S. aureus* after exposure to aerosolised  
457 formulations. (A) Complete formulation-Glycol and Ethanol; (B): Glycol only  
458 formulation; (C) Ethanol only formulation; (D): Blank formulation. Bacteria  
459 were coloured using the GNU Image Manipulation Program (GIMP) version  
460 2.8.22.

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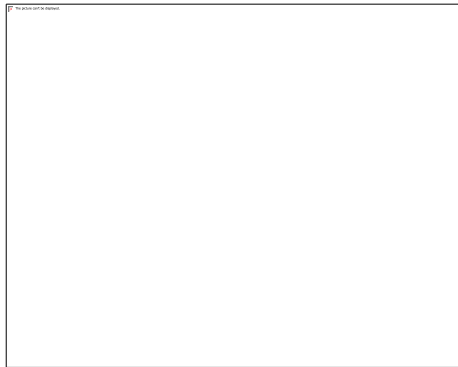
462 (A)



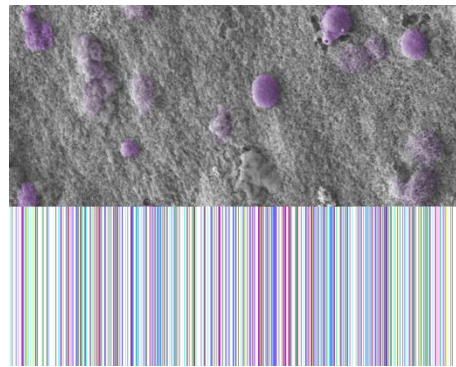
(B)



468 (C)



(D)



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479 **Figure 5** SEM images of *E. coli* after exposure to aerosolised formulations.

480 (A) Complete formulation-Glycol and Ethanol; (B): Glycol only formulation; (C)

481 Ethanol only formulation; (D): Blank formulation. Bacteria were coloured using

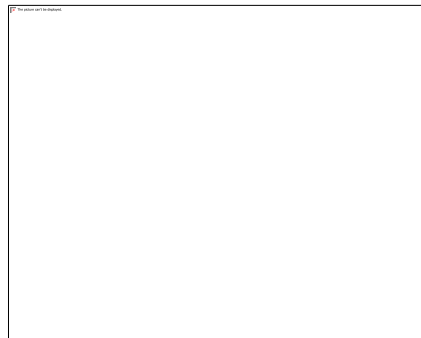
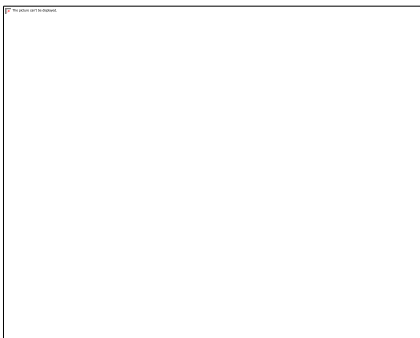
482 the GNU Image Manipulation Program (GIMP) version 2.8.22.

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484 (A)

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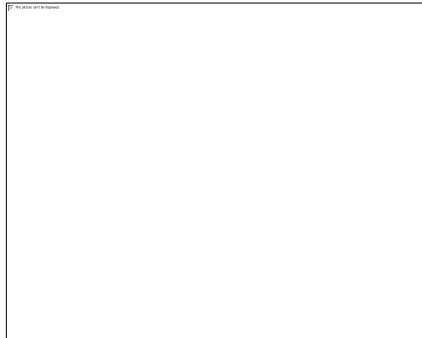
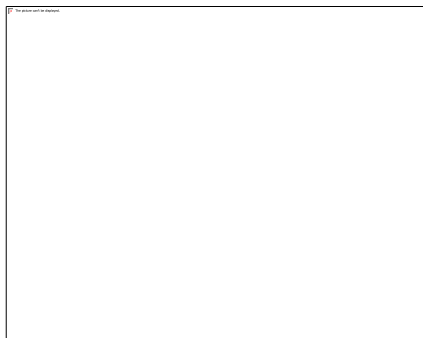
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490 (C)

(D)

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499 **Figure 6** SDS-PAGE analysis of outer membrane proteins. Line 1: Standard  
500 molecular weight; line 2: control *E. coli* without aerosolized  
501 formulation; line 3. *E. coli* treated with the blank formulation (D) and  
502 line 4: *E. coli* treated with the glycol and ethanol formulation (A).

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