1 **TITLE PAGE**

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3 **Article title**

4 Comparative effect of seven prophylactic locks to prevent biofilm biomass and viability in

5 intravenous catheters

6

7 **Short running title**

8 Prophylactic intravenous catheter locks

9

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Transparency declarations

None to declare.

ABSTRACT

Background

Patients requiring long-term intravenous access are at risk of intraluminal catheter bloodstream infection. 'Prophylactic' locks aim to limit this risk but there is uncertainty regarding the most effective lock.

Objectives

This study aimed to develop a novel technique intended to replicate clinical procedures to compare the effectiveness of various 'prophylactic' locks against biofilm biomass ('biomass') formation and biofilm viability ('viability') of *Escherichia coli* and *Staphylococcus epidermidis* in intravenous catheters.

Methods

For ten consecutive days 10^6cfu/mL *E. coli* NCTC 10418 and *S. epidermidis* ATCC 12228 were separately cultured in single lumen 9.6 French silicone tunnelled and cuffed catheters. These were flushed with 0.9% w/v sodium chloride using a push-pause technique before and after instillation of seven 'prophylactic' locks (water, ethanol, sodium chloride, heparinised sodium chloride, citrate, taurolidine plus citrate, and taurolidine; each in triplicate) for 6 hours daily. Intraluminal 'biomass' and 'viability' were quantified using crystal violet staining and flush culture respectively.

Results

The reduction of ‘biomass’ and ‘viability’ depended on both agent and species. Citrate was least effective against *E. coli* ‘viability’ and ‘biomass’ but most effective against *S. epidermidis* ‘viability’, and taurolidine was most effective against *E. coli* ‘biomass’ and ‘viability’ but least effective against *S. epidermidis* ‘viability’. ‘Biomass’ and ‘viability’ were significantly correlated in *E. coli* between ($r=0.997$, $P<0.001$) and within ($r=0.754$, $P=0.001$) interventions, but not in *S. epidermidis.*

Conclusions

A novel technique found the effect ‘prophylactic’ agents in reducing ‘biomass’ and ‘viability’ varied by species. The choice of agent depends on the most likely infecting organism.

74 **INTRODUCTION**

75

76 Despite strict preventative measures, administration of medicines by the intravenous route
77 can be complicated by intraluminal catheter bloodstream infection (CBI). CBI involves
78 adherence of bacteria (or fungi) to one another on the internal surface of the catheter to form
79 a biofilm, which can reduce the effectiveness of treatment. The microbe responsible for an
80 episode of CBI varies, although the gram-negative *Escherichia coli* and the gram-positive
81 *Staphylococcus epidermidis* or other coagulase negative staphylococci are common, if not the
82 most common causes of CBI. For example, *E. coli* accounted for 20.5% of 264,901 blood
83 culture isolates from 45 countries[1] and coagulase negative staphylococci accounted for 31.3%
84 of 20,978 bloodstream infections in 42 US hospitals.[2]

85

86 Whilst the current variability between different definitions of CBI affects reported rates,[3]
87 published frequencies are as high as five percent or more of patients or catheters. For
88 example, CBI affected 5.2% of adult Korean cancer patients with peripherally inserted central
89 catheters,[4] 5.1% of nearly eight million US hospital admissions with sepsis,[5] and 6.1% of
90 catheters of UK inpatients receiving parenteral nutrition.[6] CBI can cause patients to become
91 distressed and miss treatments, while increasing costs from more investigations, additional
92 treatments and longer length of hospital stay. For example, one episode of CBI is reported to
93 cost 9,900 GBP in England (in 2012)[7] and as much as 56,167 USD (in 1998) in US critically
94 ill surgical patients.[8]

95

96 Episodes of CBI are usually treated soon after they are clinically detected, but 'prophylactic'
97 locks that aim to prevent CBI in long-term venous lines are also used. These are often
98 prescribed for patients with a history of repeated episodes of CBI, especially when adequate

99 aseptic techniques have already failed to prevent CBI. They may also be prescribed when the
100 repeated episodes of CBI are either due to the same microbial species or to different
101 microbial species arising from the same persistent source, such as intra-abdominal sepsis.
102 Some prophylactic locks are also used to maintain open venous access when multiple other
103 veins are occluded. However, there is uncertainty and confusion about the choice of
104 'prophylactic' lock, especially since it varies between institutions, and between different
105 groups of patients in the same institution (e.g. patients receiving home parenteral nutrition
106 and haemodialysis). The 'prophylactic' agent prescribed in an institution may depend on
107 historic practice, the presence of specific antimicrobial resistance, and the likelihood of
108 encountering certain microbes. The general lack of studies in this field has resulted in a poor
109 evidence base and continued uncertainty about 'prophylactic' agent selection. Another
110 general issue is that although many microbial characteristics, including their sensitivity to
111 anti-microbial agents, have arisen from studies of planktonic cells, CBI is usually associated
112 with biofilms on catheters, which may protect the microbes against host defences and
113 antimicrobial agents. Therefore, studies examining the effectiveness of interventions to
114 prevent or treat CBI should include biofilms.
115
116 This laboratory study aimed to examine and compare the effectiveness of a number of
117 'prophylactic' interventions (agents used in intravenous catheters as locks) to guard against
118 viability of *E. coli* and *S. epidermidis* in biofilms. The study also aimed to examine
119 relationships between the microbial biofilm biomass and viability within biofilms, using
120 methodology that is relevant to routine clinical practice.
121

122 **MATERIALS AND METHODS**

123

124 An examination was made of the relative effectiveness of seven interventions (Table 1) as
125 prophylaxis against the formation of biofilm in intravenous catheters by each of *E. coli*
126 NCTC 10418 and *S. epidermidis* ATCC® 12228™ over a ten-day period. All manipulations
127 were undertaken under aseptic technique using products within their expiry date. All
128 catheters were single lumen 9.6 French silicone tunnelled and cuffed (Bard Medical,
129 Crawley, UK) that were flushed with 2mL sterile 0.9% w/v sodium chloride before and after
130 each use with a push-pause method. Standard needles were used with luer lock syringes
131 except for drawing up from glass ampoules when 5-micron filter needles were used. All of
132 the interventions were prepared by a single operator experienced in aseptic medicine
133 compounding (author PDA) and all other practical manipulations were undertaken by two
134 operators (authors PDA and PS).

135

136 **Exposure of bacteria to the interventions**

137

138 Each day for ten consecutive days, solutions containing 10^6cfu/mL of each of *E. coli* and *S.*
139 *epidermidis* in tryptic soy broth were freshly prepared and locked in intravenous catheters
140 (N=21 per species; total N = 42) for 18 hours at a controlled temperature of 37.0°C (range
141 35.5-37.0°C) whilst being gently shaken 100 times per minute. At the end of the 18-hour
142 period each catheter was flushed to expel the prophylactic agent and the relevant intervention
143 solution was locked for 6 hours in the catheters, which were kept out of direct sunlight in an
144 air-conditioned room at an ambient temperature range of 19.2-26.6°C. At the end of the 6-
145 hour period each catheter was flushed with 0.9% w/v sodium chloride to expel the

prophylactic agent. This 24-hour procedure was repeated 10 times, after which the catheters were sampled for both biofilm biomass and viability (see below).

Testing for intraluminal biofilm biomass

After the final flush on day 10 (see above), which was used to clear and discard the 'locked' intervention solution (and a further identical flush for biofilm viability testing - the 'viability flush' - see below), a 5mL flush of air was used to expel any remaining solution from the catheter. Then a 0.01% v/v crystal violet dye in sterile distilled water was used to fill the catheter lumen and left there in situ for 10 minutes to allow the dye to bind to any intraluminal biofilm biomass. The excess dye was removed by flushing the catheter with sterile distilled water until the liquid expelled from the catheter tip (which was discarded) became clear. In turn the sterile distilled water within the catheter lumen was expelled (and discarded) using a 5mL flush of air. Next, 30% v/v acetic acid was used to fill the catheter lumen, and then left in situ for 10 minutes to extract dye from any intraluminal biofilm biomass. Finally, this was flushed out by 5mL air so that the coloured fluid could be collected in a sterile 7mL bijou for absorbance measurements at 550nm[9] using 30% v/v acetic acid in distilled water reference. Samples with high readings were diluted with 30% v/v acetic acid in distilled water as required. Three fresh catheters which were not exposed to *E. coli* or *S. epidermidis* were subjected to the same procedure as the catheters exposed to bacteria. They acted as 'controls', to take into account any binding of dye to the catheter. The optical density (measured at 550nm; OD_{550}) obtained after subtracting the 'control' value from the 'test' value, was taken to be the marker for biofilm biomass.

Testing for intraluminal biofilm viability

171

172 Part of the 'viability catheter flush' obtained on day 10 (see preceding section) that had been
173 collected was mixed with an equal volume of sterile tryptic soy broth (0.75mL each; total
174 volume 1.5mL) and incubated aiming for a nominal 37°C (actual range 35.5-36.0°C) for 24
175 hours before absorbance was measured at 600nm (OD_{600}) against a tryptic soy broth
176 reference (none of the samples required dilution immediately prior to testing). This result was
177 taken to be the marker for biofilm viability.

178

179 **Sensitivity analysis**

180

181 In comparing *E. coli* and *S. epidermidis* it was assumed that the same optical density readings
182 in the two species represented the same 'biomass' (measured at 550nm; OD_{550}) and
183 'viability' (measured at 600nm; OD_{600}) (i.e. ratio of *S. epidermidis*/*E. coli* = 1.0). To reduce
184 uncertainty two sensitivity analyses were carried out: one in which the ratio (*S. epidermidis* to
185 *E. coli*) of biomass at a given OD_{550} varied from 0.75 to 1.25, and the other in which the ratio
186 of 'viability' at a given OD_{600} varied from 0.50 to 2.0. A direct single comparison of viability
187 was also undertaken at OD_{600}.

188

189 **Statistics**

190

191 The effects of the interventions were examined using a general linear model employing
192 heteroscedasticity-consistent 3 (HC3) standard error methodology. Biofilm biomass or
193 viability were separately included as the dependent variable with species (*E. coli/S.*
194 *epidermidis*) and intervention as fixed factors. The full factorial model, with the species-
195 intervention interaction, was used to examine the effect of different interventions within

196 species as well as the effect of the same intervention across species. Multiple t-test
197 comparisons are reported with Bonferroni corrections.
198
199 The relationship between biofilm biomass and viability within each species was examined in
200 two ways: by correlating the average values associated with each intervention (between
201 intervention group correlation), and by correlating individual values using ANCOVA to
202 establish the repeated measures correlation (within intervention group correlation).[10]
203
204 Statistical analyses were undertaken using version 25 of the software SPSS (IBM; Chicago,
205 Illinois, USA), and unless otherwise stated data reported as mean±SEM and $P<0.05$
206 considered significant.
207
208 **RESULTS**
209
210 All the results are presented after subtracting the measurements on the control samples, which
211 had not been exposed to bacteria, from those of samples that had been exposed to bacteria.
212 For biofilm biomass, which involved measurement of crystal violet at OD_{550}, this meant
213 subtracting the mean control value of 0.171 (0.171±0.002; n=3).
214
215 Figure 1 is used to illustrate three aspects of the effects of interventions on biofilm biomass
216 and viability. First, all interventions combined produced a more than 5.5-fold greater biofilm
217 biomass using *E. coli* than *S. epidermidis* (OD_{550} of the eluted crystal violet; 8.432±0.448 v
218 1.502±0.153, $P<0.001$), and a 7-fold greater biofilm viability (OD_{600} of organism eluted from
219 the catheter after 10 days of intervention; 8.522±0.440 v 1.226±0.077, $P<0.001$).
220 Furthermore, with the exception of water for injection, all the interventions produced

221 significantly greater biomass ($P<0.001$, Bonferroni corrected) and viability ($P<0.001$, but for
222 2% taurolidine $P=0.004$, Bonferroni corrected) in *E. coli* than *S. epidermidis*. In the
223 sensitivity analyses, these remained highly significant ($P\leq0.001$) except in the case of biofilm
224 biomass exposed to ethanol, in which the significance was less strong ($P=0.011$, Bonferroni
225 corrected) when the ratio of *S. epidermidis/E. coli* biomass at given OD_{550} was 1.25, and for
226 both biofilm biomass and viability after water for injection, in which the results were largely
227 non-significant. The single comparison of biofilm viability between *S. epidermidis* and *E.*
228 *coli* (per 1 OD_{600}) yielded a ratio of 0.78 ($2.48x10^8$ v $3.16x10^8$), well within the range of 0.5-
229 2.0 used in the sensitivity analysis. Second, the interventions produced widely different
230 effects on biofilm biomass and viability within each genera. For *S. epidermidis*, the biofilm
231 biomass (estimated using absorbance at 550nm) varied from 0.397±0.029 (2% w/v
232 taurolidine) to 2.432±0.666 (70% v/v ethanol in water for injection) and the biofilm viability
233 (estimated using absorbance at 600nm) from 0.004±0.000 (46.7% w/v citrate) to 1.861±0.183
234 (10i.u./mL heparin in 0.9% w/v sodium chloride). For *E. coli*, the biofilm biomass varied
235 from 3.321±0.312 (2% w/v taurolidine) to 12.143±0.657 (46.7% w/v citrate) and biofilm
236 viability from 3.393±0.318 (2% w/v taurolidine) to 12.603±0.820 (46.7% w/v citrate). Third,
237 the type of organism was found to have a considerable impact on the pattern of effects
238 produced by different interventions (the significant intervention-genera interaction is
239 indicated by the divergent dotted lines on the graph). For example, the difference in biofilm
240 biomass between *E. coli* and *S. epidermidis* was found to be several times greater following
241 46.7% w/v citrate instillations than 2% w/v taurolidine instillations. The same was found for
242 biofilm viability.
243
244 Close examination of Figure 1 also shows that with *E. coli* the pattern of biofilm biomass
245 established by the various interventions is mirrored by the pattern for biofilm viability.

246 However, this is not the case for *S. epidermidis*. These observations are more directly and
247 clearly illustrated in Figure 2 (*upper*) which shows the correlation between biofilm biomass
248 and viability using average values for each intervention (i.e., correlation between intervention
249 groups). A strong and significant correlation was found for *E. coli* (*upper left*; r=0.997,
250 P<0.001) but not for *S. epidermidis* (*upper right*; r=0.407, P=0.365). This is mirrored by the
251 repeated measures correlation (correlation within intervention groups) shown in Figure 2
252 (*lower*), which was also found to be significant for *E. coli* (*lower left*; r = 0.754, P=0.001) but
253 not *S. epidermidis* (*lower right*; r= 0.145, P=0.606).
254

DISCUSSION

This laboratory study has provided insights into the characteristics of *E. coli* and *S. epidermidis* biofilms established within intravenous catheters that are used in clinical practice, as well as insights into the effects of interventions that aim to prevent biofilm formation in clinical practice. The novel methodology employed involved techniques used in clinical practice such as push-pause flushing of complete intravenous catheters, rather than those not used in clinical practice (for example, see below for discussion on the use of wells).

Overall, it appears that after exposure of intravenous catheters to a variety of interventions and the same density of *E. coli* and *S. epidermidis* over the same period of time, the *E. coli* biofilm was several times more plentiful than the *S. epidermidis* biofilm, producing highly significant differences even at the extremes of the sensitivity analyses. This observation, affecting both biofilm biomass and viability, was also found in a study of biofilms involving polystyrene wells,[11] although another study using polystyrene wells[12] found that biofilm biomass was greater with *S. epidermidis* than *E. coli*. The discrepancies between studies may be due to methodological differences. In general, laboratory studies use methodology unrelated to clinical practice, such as those examining the effects of interventions in wells, rather than in catheters, using unrealistically small surface areas and volumes of antimicrobial agents, often over shorter periods of time than would typically apply in routine clinical practice. In addition, laboratory studies generally do not account for the catheter flushing that frequently occurs in clinical practice, although efforts to do so were made in the present study using daily flushes for 10 days. The use of different strains of *E. coli* and *S. epidermidis* in the above-mentioned studies may have also contributed to the discrepancies. In addition, it is known that multiple regulatory factors are involved in biofilm formation, but these differ

280 between *E. coli*[13] and *S. epidermidis*.[14] For example, in *E. coli* several factors regulating
281 biofilm formation concern its motility, which can increase interaction between the bacteria
282 and a surface,[13] but these are not relevant to *S epidermidis* which is non-motile. In *S.*
283 *epidermidis* an important regulatory factor is polysaccharide intercellular adhesin (PIA). Its
284 presence has general relevance to biofilms produced by this species, including those
285 subjected to a high shear stress (catheter lumen),[14] since without PIA many isolates are
286 unable to form biofilms.
287
288 This present study also found that after exposure of catheter biofilms to various prophylactic
289 interventions biofilm biomass and viability not only differed widely within species, but the
290 overall pattern also differed widely between species (Figure 1). This is not surprising since
291 different genera have distinct metabolic machineries that respond differently to external anti-
292 microbial interventions, including antibiotics. However, the observations raise a practical
293 clinical difficulty since a microbial agent that is effective prophylactically against one species
294 is not necessarily effective against another species that may be just as likely to cause CBI.
295 For example, citrate was found to be the least effective agent against both the biomass and
296 viability of the *E. coli* biofilm and most effective against the viability of *S. epidermidis*
297 biofilm and second most effective against its biofilm biomass formation. In contrast,
298 taurolidine was the most effective against both the biofilm biomass and viability of *E. coli*,
299 and least effective against the biofilm viability of *S. epidermidis*, although taurolidine was
300 also among the most effective agents against its biofilm biomass. Therefore, a potentially
301 fruitful avenue for further investigation would be prescription of prophylactic intervention
302 according to microbial target rather than according to cohort. Another possibility could be the
303 use of a combination of agents to prevent biofilm formation, although such studies would

304 also need to consider issues such as stability, sterility, practicality, and development of anti-
305 microbial resistance.
306
307 Another key observation is the strong relationship between biofilm biomass and viability in
308 *E. coli* (see Figure 2 for relationships between and within interventions), which contrasts with
309 the weak nonsignificant relationship in *S. epidermidis.* The reason for this difference is
310 unclear and many factors are probably involved in causing the biofilm biomass-viability
311 dissociation in *S. epidermidis*. For example, since crystal violet stains both living and
312 dead/dying cells it is possible that the proportion of these may vary after different
313 interventions. It is also theoretically possible for cells to be 'viable' but not 'culturable', but
314 this was not investigated in the present study. In addition, it is known that *S. epidermidis*
315 biofilms contain cells growing aerobically or fermentatively, as well as cells that are dead or
316 dying.[15] Since crystal violet staining was not undertaken in this study[15] it was not possible to
317 ascertain whether these cells stain with different intensity. Furthermore, it can be
318 hypothesised that the process by which individual cells or intact sections of *S. epidermidis*
319 biofilms slough off[14] or wash away during catheter flushes involves different proportions of
320 living to dead/dying cells that could contribute further to the dissociation between biofilm
321 biomass and viability.
322
323 This study has some limitations. Apart from those already discussed, the present
324 methodology does not account for the clinical exposure of the catheter to blood or any
325 interactions of the biofilm with host cells and other circulating substances. Also, since the
326 study did not examine the characteristics of any biofilm that may have been shed during
327 catheter flushing (apart from its viability on day 10), the measurements of biofilm biomass
328 and viability relate only to the final status of the biofilm, and not necessarily to the total

329 biofilm formation over the 10-day period of study. In addition, care should be taken not to
330 extrapolate the results of the present study to other bacteria or fungi, or other types of
331 catheter. Nevertheless, the novel methodology employed in this study can be used together
332 with other investigative techniques to further explore the biology of CBI and methods to
333 prevent and treat it.
334

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348 **TRANSPARENCY DECLARATIONS**

349

350 None to declare.

351

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394

395 **Table 1** The seven interventions tested as prophylaxis against biofilm formation of

396 *Escherichia coli* and *Staphylococcus epidermidis* in intravenous catheters

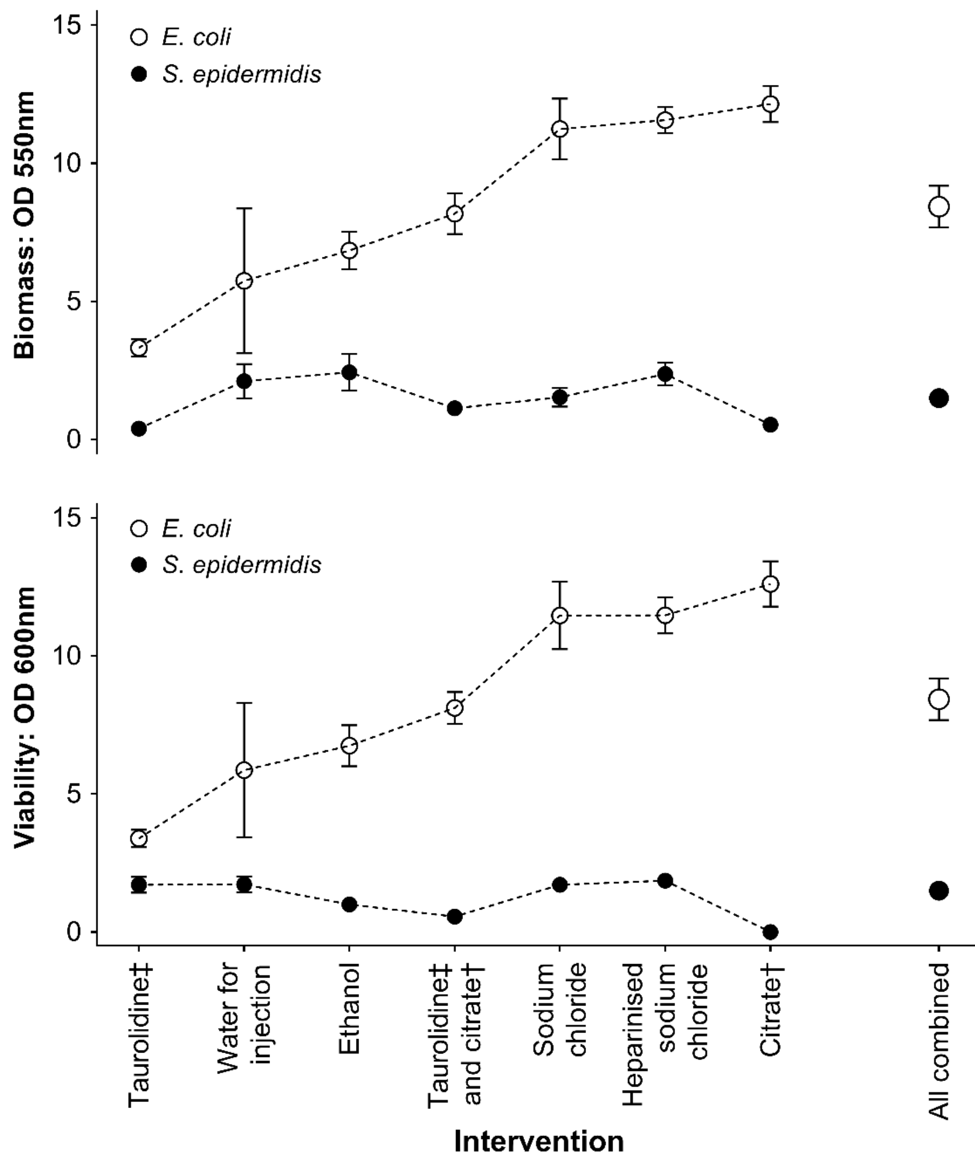
Intervention	Agent	Brand name	Manufacturer
1	Water for injection	Generic product	B. Braun, Melsungen, Germany
2	70% v/v ethanol in water for injection	Not applicable	Sigma-Aldrich Company Ltd., Gillingham, England[a]
3	0.9% w/v sodium chloride	"Posiflush"	BD, Drogheda, Ireland (10mL size)
4	10i.u./mL heparin in 0.9% w/v sodium chloride	Generic product	Wockhardt, Wrexham, Wales
5	46.7% w/v citrate	"Citralock-S"	s.a.l.f.s.P.a. Laboratorio Farmacologico, Bergamo, Italy
6	1.35% w/v taurolidine and 4% w/v citrate	"Taurolock Classic"	TauroPharm GmbH, Waldbüttelbrunn, Germany
7	2% w/v taurolidine	"Taurosept"	Geistlichpharma AG, Wolhusen, Switzerland

397 [a] The 70% v/v ethanol in water for injection was prepared under aseptic technique by diluting

398 absolute alcohol (drawn up through a 0.2 micron disc filter) with water for injection

399 (intervention 1).

400



401

402 **Figure 1** The effect of seven 'prophylactic' interventions on the biofilm biomass (*upper*

403 *figure*) and viability (*lower figure*) of *Escherichia coli* (open circles) and *Staphylococcus*

404 *epidermidis* (closed circles) elicited on day 10 after the start of the interventions. Individual

405 interventions are shown in small circles and the averages of all interventions in large circles.

406 The divergent dotted lines indicate a significant species-intervention interaction. The standard

407 errors of the mean for some interventions involving *S. epidermidis* are small and hidden

408 behind the symbols. All pairwise comparisons are highly significant to varying degrees (with

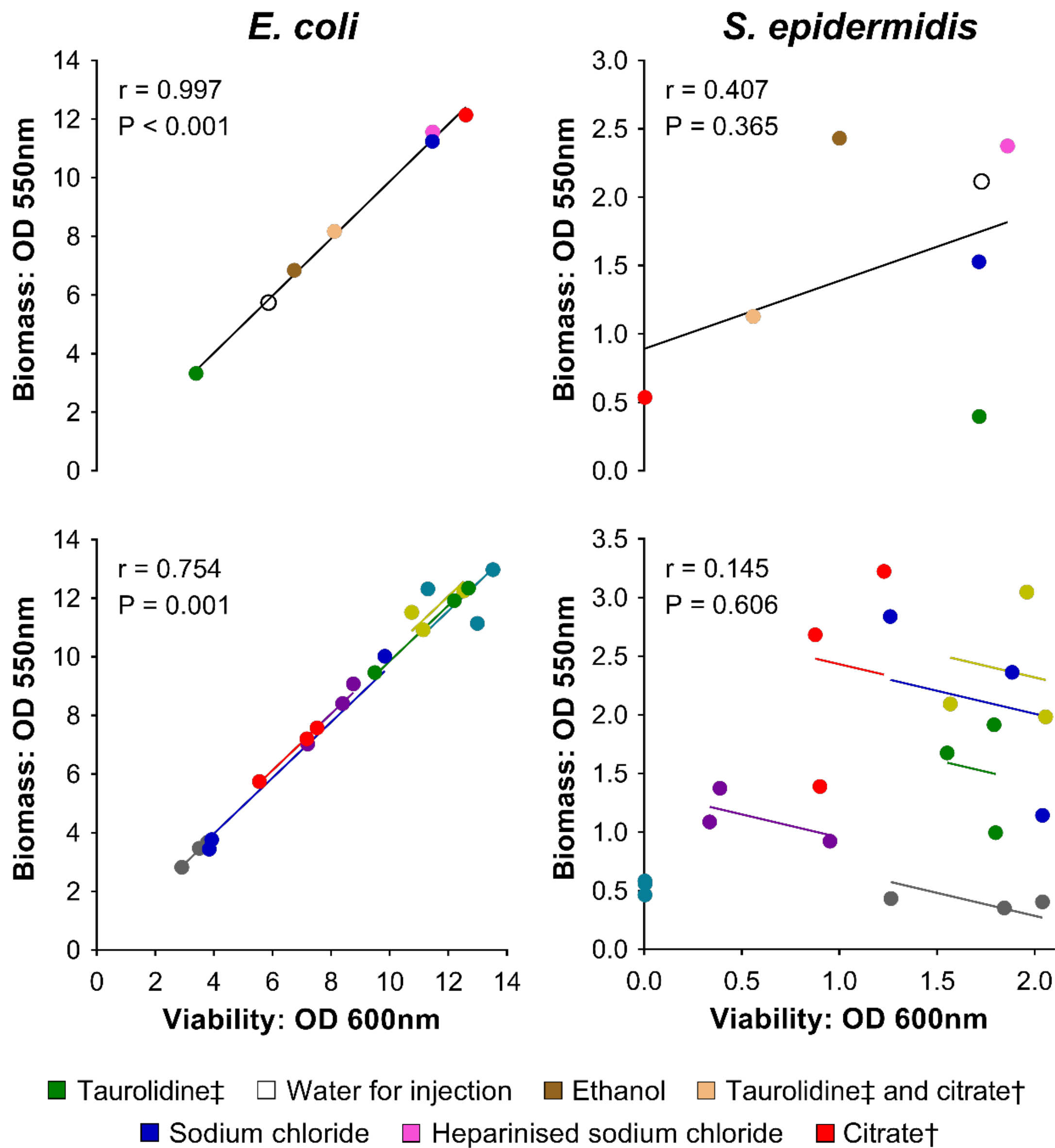
409 and without Bonferroni adjustments) with the exception of water for injection, which was not

410 significant. The concentrations of taurolidine (‡) and citrate (†) in the interventions indicated

411 differ (Table 1). Data are presented as mean±SEM and OD = optical density measured at

412 550nm for biofilm biomass and at 600nm for biofilm viability.

413



414

415 **Figure 2** The relationship between biofilm biomass and viability in *Escherichia coli* (*left*)
416 and *Staphylococcus epidermidis* (*right*) on day 10 after administration of 'prophylactic'
417 agents. The *upper* graphs show the between group correlation (using averaged values; n=3 for
418 each invention group for each species) and the *lower* graphs the within group correlation
419 (repeated measures correlation using the individual values; total N=21 for each species). The
420 concentrations of taurolidine (‡) and citrate (†) in the interventions indicated differ (Table 1).
421 OD = optical density measured at 550nm for biofilm biomass and at 600nm for biofilm
422 viability.