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Disinfectant Efficacy Against Dry Surface Biofilms of Staphylococcus Aureus and Pseudomonas Aeruginosa Is Product, Time Point and Strain Dependent

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Abstract

Background:

Globally, healthcare associated infections (HAI) are the most frequent adverse outcome in healthcare delivery. Although bacterial biofilms contribute significantly to the incidence of HAI, few studies have investigated the efficacy of common disinfectants against dry surface biofilms (DSB). The objective of this study was to evaluate the bactericidal efficacy of seven disinfectants against DSB of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. We hypothesized that overall, hydrogen peroxides, sodium dichloro-s-triazinetrione and quaternary ammonium compounds plus alcohol disinfectants will be more bactericidal against DSB than quaternary ammonium. We also hypothesized that regardless of differences in product chemistries, higher bactericidal efficacies against DSB will be exhibited after 24 h of dehydration compared to 72 h.

Methods: Wet surface biofilms of *S. aureus* and *P. aeruginosa* were grown following EPA-MLB-SOP-MB-19 and dehydrated for 24 h and 72 h to establish DSB. Seven EPA-registered disinfectants were tested against dehydrated DSB following EPA-MLB-SOP-MB-20.

Results: Overall, quaternary ammonium plus alcohol, sodium dichloro-s-triazinetrione, and hydrogen peroxide products were more efficacious against DSB than quaternary ammoniums for both tested strains. While there was no significant difference in biofilm killing efficacies between 24 h and 72 h *S. aureus* biofilms, significantly higher $\log_{10}$ reductions were observed when products were challenged with 24 h *P. aeruginosa* DSB compared to 72 h *P. aeruginosa* DSB.

Conclusion: Strain type, active ingredient class, and dry time significantly impact disinfectant efficacy against DSB of *S. aureus* or *P. aeruginosa*.

Background

Healthcare associated infections (HAI), a result of diverse interactions among modern healthcare practices, hospital environments, and growing antibiotic resistance, among other factors, pose a crucial threat to human well-being [1]. Globally, the acquisition of HAI is the most frequent adverse outcome in healthcare delivery [2]. In the United States, approximately 633,300 patients are affected by 687,200 HAI [3] with more than 72,000 deaths every year [4]. In Europe, about 4.5 million HAI occur yearly in acute care hospitals [5] with about approximately 135,000 deaths [6]. In low and middle income countries (LMIC), the density of HAI in adult intensive care units is estimated at 47.9 per 1,000 patient days, which is higher than rates in the US and Europe [7]. Comparing HAI incidence rates in developed and LIMC, the incidence rate is seven out of 100 patients in developed economies and ten out of a 100 in LMIC [8].

The prevalence of HAI has been associated with biofilm formation by bacteria [9]. Bacterial biofilms are ubiquitous and represent approximately 99% of the world's known bacterial population [10]. The National Institutes of Health (NIH) of the US estimates that about 80% of all chronic infections are due to biofilm formation [9]. Biofilms are comprised of microbial cells adhered to a surface and to each other, forming a micro-colony encased in a polysaccharide dominant matrix [11]. In addition to the cells that inhabit a biofilm, DNA, proteins and biosurfactants are prevalent [12]. Bacterial biofilms are persistent on environmental surfaces due to the ability to adhere to common surfaces and the extracellular polymeric substances (EPS) they produce [13]. The EPS forms a matrix that presents a major barrier to removal from surfaces in healthcare facilities as it is "resistant" to physical stress [14] and shields underlying bacterial cells from direct contact with disinfectants [15]. As a result of the EPS matrix [11Donlan, 2000], the presence of efflux pumps and persister cells [16], bacterial biofilms are about 1,000 times less susceptible to disinfectants than their planktonic counterparts [17]. Additionally, the ability for disinfectants to penetrate the biofilm matrix is affected by the water binding characteristic of the EPS matrix [11], and pH differences among various layers of biofilms [18]. These features may result in the aggregation of organic acids leading to the deactivation of less potent disinfectants that may be non-lethal [18].

Bacterial biofilms have the ability to develop and persist for up to 12 months [19] on wet and dry surfaces in the hospital environment despite repeated cleaning [20]. Dry surface biofilms (DSB) are particularly widespread on surfaces in healthcare facilities [20, 21]. In a recent 2018 study by Ledwoch et al., DSB were detected in 95% of 61 samples collected from hospitals in Wales [22]. Such surfaces included commodes, clipboards and sanitizing bottles [22]. DSB have also been detected on indwelling catheters [23]. Although multi-species dry surface biofilms have been detected on a range of surfaces in healthcare facilities, major HAI pathogens as *S. aureus* [22] and *P. aeruginosa* are predominant [24, 25].

While DSB are widespread on surfaces in healthcare facilities, they are also harder to kill than wet surface biofilms [26]. This is the case as overall, DSB are characterized by a denser EPS matrix than wet surface biofilms [26, 27]. Moreover, with prolonged desiccation and starvation of bacteria in DSB, there is an increase in the overall percentage of protein content and slightly decreased carbohydrate content compared to wet surface biofilms [26, 28] Being the principal component of biofilms, this increase in the proportion of proteins may further contribute towards the reduced bactericidal efficacy of disinfectants against DSB. [29]. Biofilms may survive longer due to metabolic changes that may result from cell-cell signaling and from the presence of a biofilm matrix that facilitates nutrient recycling and transformation from lysed cells [30].

In the current protocol used by the Environmental Protection Agency (EPA) for biofilm claims on disinfectants, wet surface biofilms of *S. aureus* and *P. aeruginosa* are the required test pathogens [31]. Under real world conditions such as in healthcare facilities, disinfectants are relied on to inactivate bacteria on dry surfaces [32], which are usually in the form of DSB [26]. Despite widespread evidence that bacteria in healthcare environments are more likely to be encased in DSB, the standard test for disinfectant efficacy testing and registration with the EPA are conducted using planktonic bacteria or bacteria in wet biofilms. To the best of our findings, no studies have evaluated the bactericidal efficacy of disinfectants against dry surface biofilms of *S. aureus* and *P. aeruginosa* established at different dehydration time points consistent with routine cleaning and disinfection schedules recommended by the CDC [33]. In a previous study, our group developed a rapid model for establishing DSB of *S aureus* and *P. aeruginosa* at different time points and at mean $\log_{10}$ densities sufficient for disinfectant efficacy testing [34]. In this study, we evaluated the bactericidal efficacy of seven liquid disinfectants against DSB of *S. aureus* and *P. aeruginosa* after 24 h and 72 h of dehydration. We hypothesized that overall, hydrogen peroxide, sodium dichloro-s-triazinetrione, and quaternary ammonium compounds plus alcohol disinfectants will be more bactericidal against DSB than quaternary ammonium disinfectants based on our prior work. We also hypothesized that regardless of differences in product chemistries, higher bactericidal efficacies against DSB will be exhibited after 24 h of biofilm dehydration compared to 72 h of dehydration.

Methods

Bacteria strains and disinfectants tested in this study

DSB of *S. aureus* ATTC-6538 and *P. aeruginosa* ATCC-15442 were established on borosilicate glass coupons (1.27 ± 0.013 cm; Biosurface Tech, Inc.) following Nkemngong et al., 2020 [34]. These strains were selected as they are standard strains of choice for disinfectant efficacy testing [31]. They are also the standard EPA strains for registering disinfectants with claims against wet surface bacterial biofilms [35].

Table 1 Active ingredients and contact times for disinfectants tested in this study.

Disinfectant product[a]	Disinfectant Active Ingredient(s)[d]	Dilution at use	Active level at use[e]	Label contact time (mins)[f]
CL[b]	48.21% sodium dichloro-s-triazinetrione	RTU[d]	4,306 ppm	4
SH	0.39% sodium hypochlorite	RTU	0.39%	1
HP1	0.5% hydrogen peroxide	RTU	1.4%	1
HP2	0.5% hydrogen peroxide	RTU	0.5%	1
QA1[c]	0.25% n-alkyl (68%C_{12}, 32%C_{14}) dimethyl ethylbenzyl ammonium chloride 0.25% n-alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) dimethyl benzyl ammonium chloride 55% isopropanol	RTU	0.5% [g] + 55%	2
QA2	0.76% didecyldimethyl ammonium chloride 15% isopropanol 7.50% ethanol	RTU	0.76% [g] + 22.5%	1
QT [c]	0.14% n-alkyl (68%C_{12}, 32%C_{14}) dimethyl ethylbenzyl ammonium chloride 0.14% n-alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) dimethyl benzyl ammonium chloride	RTU	0.28%	3

[a] Abbreviated naming scheme for commercially available EPA registered disinfectants used in this study;

[b] Tablet, ready-to-use (RTU) concentration prepared following manufacturer guidelines;

[c] Liquid expensed from RTU disinfectant wipes;

[d] RTU concentration;

[e]Active ingredient concentration after dilution;

[f] Defined label contact time;

[g] Total quaternary ammonium plus alcohol content.

DSB establishment on borosilicate glass coupons

DSB of *S. aureus* ATTC-6538 and *P. aeruginosa* ATCC-15442 were established on borosilicate glass coupons (1.27 ± 0.01 cm; Biosurface Tech, Inc., Bozeman, MT) following Nkemngong et al., 2020 [34]. Wet surface biofilms were established following EPA-MLB-SOP-MB-19 through batch and continuous stir tank reactor (CSTR) phases [35]. The batch medium was 3.0 g/L TSB for *S. aureus* and 300 mg/L TSB for *P. aeruginosa*. A 500 ml batch medium held in a CDC biofilm reactor (Biosurfaces Technologies, Inc., Bozeman, MT) was inoculated with one ml of an overnight culture of *S. aureus* or *P. aeruginosa*. The batch phase lasted 24 ± 2 h with the CDC biofilm reactor (Biosurfaces Technologies, Inc., Bozeman, MT) mounted on a magnetic hot plate stirrer (Talbays, Thorofare, NJ) set at 60 ± 5 rpm at 36 ± 1°C for *S. aureus* or 125 ± 5 rpm at 21 ± 2°C for *P. aeruginosa*. CSTR medium in 20 L of sterile distilled water had a final concentration of 1.0 g/L TSB for *S. aureus* and 100 mg/L TSB for *P. aeruginosa*. CSTR medium was continuously pumped through the CDC biofilm reactor for 24 ± 2 h for both strains.

After wet surface biofilms were established through the batch and CSTR phases, rods from the CDC biofilm reactor; each holding three borosilicate glass coupons were dehydrated for 24 h and 72 h at 25°C or 21°C for *S. aureus* and *P. aeruginosa,* respectively. Dry times and dehydration temperatures were informed by Nkemngong et al., 2020 [34].

Disinfectant efficacy testing against DSB of *S. aureus* and *P. aeruginosa*

At each dehydration time point (24 h and 72 h), disinfectants (Table 1) were tested against DSB following EPA-MLB-SOP-MB-20 [35]. At each dry time and for each disinfectant product, three coupons with DSB of *S. aureus* or *P. aeruginosa* were harvested into sterile 50 ml conical tubes. Three coupons each after 24 h and 72 h of dehydration served as negative controls; controls were treated with four ml of phosphate buffer saline (PBS) with a one-minute contact time. At each of the dry times, three harvested coupons (technical replicates) were independently treated with four ml of each disinfectant (Table 1). At the label-defined contact time of each disinfectant product and PBS, 36 ml of neutralizing buffer (1L H_2O + 5.2 g Difco neutralizing buffer; Becton, Dickinson and Company Sparks, MD) was added to each treated coupon to stop the disinfectant action. Treated coupons were vortexed and sonicated to release DSB from coupons into solution as described by Nkemngong et al., 2020 [34]. Post treatment with disinfectants, DSB of *S. aureus* or *P. aeruginosa* were vacuum-filtered onto filter membranes following EPA-MLB-SOP-MB-20 [35]. Negative controls were spread plated following EPA-MLB-SOP-MB-20 [35]. Eight biological replicates were completed for QA and QT products and five biological replicates for CL, SH and HP products as informed by Lineback et al., 2018 [36].

Statistical analysis

Log_{10} reductions resulting from the treatment of coupons with DSB were calculated and used for statistical analyses. Specifically, mean bacterial log_{10} densities per coupon were calculated for disinfectant and PBS-treated coupons. Mean log_{10} densities per disinfectant-treated coupon were normalized against the mean log_{10} densities of control coupons to determine log_{10} reductions. The least squares method of the PROC GLIMMIX procedure was used to analyze and compare mean log_{10} reductions (n=70 per strain; N=140; α=0.05) among the seven tested disinfectant products. The same test was used to statistically compare mean log_{10} reductions at 24 h and 72 h. Pair-wise comparisons among products, strains, and dry times were completed with Tukey adjustments. All statistical procedures were completed using SAS version 9.4 (SAS Institute, Cary, NC).

Results

Dry time does not significantly impact disinfectant efficacy against *S. aureus* DSB, unlike *P. aeruginosa* DSB

Mean log_{10} densities of *S. aureus* DSB per control coupon after 24 h and 72 h of dehydration were 7.64 ± 0.76 and 7.00 ± 0.89, respectively. On average, the bactericidal efficacies for all tested disinfectants against *S. aureus* DSB at 24 h and 72 h dehydration time points were 6.30 ± 1.27 and 5.89 ± 1.21, respectively. There was no significant difference between the average log_{10} reductions per coupon of *S. aureus* DSB at 24 h compared to 72 h ($P>0.05$; Figure 1).

The average log_{10} densities of *P. aeruginosa* DSB per coupon pre-treatment were 7.40 ± 0.75 and 6.77 ± 0.61 after 24 h and 72 h dry times, respectively. There were no significant differences between the average log_{10} density per coupon after 24 h and 72 h of dehydration (P□0.005). On average, the mean log_{10} reduction per coupon for all tested disinfectants after 24 h and 72 h were 5.50 ± 1.45 and 4.65 ± 1.63, respectively.

Overall and regardless of the product type or active ingredient class, significantly higher bactericidal efficacies against DSB of *P. aeruginosa* were recorded after 24 h compared to 72 h of dehydration (P<0.05; Figure 1).

Bactericidal efficacy against *S. aureus* DSB varies by product type and active ingredient class

The average $\log_{10}$ densities of *S. aureus* DSB per coupon pre-treatment after 24 h and 72 h dry times were 7.64 ± 0.76 and 7.0 ± 0.89, respectively. Overall, there were significant differences among the product types and active ingredient classes (P<0.05; Figure 2). Irrespective of the dry time (24 h or 72 h), $\log_{10}$ reduction for QA2 (6.11 ± 1.30), HP2 (6.18 ± 1.72), HP1 (6.34 ± 1.154), SH (6.45 ± 0.87), CL (6.57 ± 1.11), and QA1 (6.61 ± 0.80) resulted in significantly higher $\log_{10}$ reductions than QT (6.12 ± 1.30) (P<0.05; Figure 2). However, there were no statistically relevant differences among the mean $\log_{10}$ reductions per coupon for QA1, QA2, CL, SH, HP1 and HP2 (P>0.05; Figure 2).

Overall, the mean $\log_{10}$ reduction per coupon per product type were QT (4.85 ± 0.86), HP (6.19 ± 1.43), QA (6.36 ± 1.10), SH (6.45 ± 0.87), and CL (6.57 ± 1.11) (Figure 3). QA, CL, SH and HP products were more bactericidal than the QT product (P<0.05; Figure 3). However, there were no statistically significant differences in the bactericidal efficacies between QA and HP; CL and SH, SH and QA, CL and HP, SH and HP, and CL and QA when disinfectants were challenged with *S. aureus* DSB (P>0.05; Figure 3).

Mean $\log_{10}$ reductions for *P. aeruginosa* DSB were higher for oxidizing agents compared to quaternary ammonium products

The mean $\log_{10}$ density of *P. aeruginosa* DSB per coupon was 7.08 ± 0.75 after dehydration (24 h and 72 h) and pre-treatment. Overall, product type and active ingredient class were significant (P<0.0001; Figure 2**).** Regardless of the dry time (24 h or 72 h), there were significantly higher $\log_{10}$ reductions for HP1 (5.97 ± 1.23) and HP2 (6.30 ± 1.26) as compared to QA1 (4.20 ± 0.71), QT (3.82 ± 0.53), and SH (3.23 ± 1.53) (P<0.05; Figure 2). Similarly, CL (5.79 ± 1.40) and QA2 (5.85 ± 0.87) had significantly higher $\log_{10}$ reductions against *P. aeruginosa* DSB than QA1, QT and SH (P<0.05; Figure 2**).** However, there were no statistically significant differences among QA1, QT and SH (P 0.05; Figure 2**).** There were also no statistically significant differences in the bactericidal efficacies of HP1, HP2, CL and QA2 (P 0.05; Figure 2).

There were statistically significant differences among active ingredient classes (CL, HP, SH, QA and QT (P<0.0001; Figure 3**).** Overall, HP products resulted in a significantly higher bactericidal efficacy than QT and SH products (P<0.05; Figure 3**).** Similarly, CL and QA products had significantly higher mean $\log_{10}$ reductions than QT and SH products (P<0.05; Figure 3**).** However, there were no differences between QT and SH, CL and HP, HP and QA, and QA and SH products (P 0.05; Figure 3).

Higher bactericidal efficacy against *S. aureus* DSB than *P. aeruginosa* DSB

Overall, and regardless of the product type, the bacterial strain was statistically significant (P P<0.05). The overall mean $\log_{10}$ reductions for *S. aureus* and *P. aeruginosa* were 6.096 ± 1.251 and 4.941 ± 1.505 respectively. Significantly higher $\log_{10}$ reductions were observed when the tested disinfectants were challenged with *S. aureus* compared to *P. aeruginosa* (P<0.05).

Discussion

In this study, we employed a rapid DSB model previously developed by our group for disinfectant efficacy testing and evaluated the bactericidal efficacy of seven EPA-registered disinfectants against 24 h and 72 h old DSB of *S. aureus* and *P. aeruginosa*. Specifically, we established DSB of *S. aureus* and *P. aeruginosa* at 25°C and 21°C respectively to mimic environmental conditions for the formation of DSB on dry contaminated hard non-porous surfaces in healthcare facilities.

We found that mean $\log_{10}$ densities per coupon from this study were comparable to the ranges previously reported by Nkemngong et al., 2020 [34]. We found that overall and irrespective of dry time, CL, SH, HP and QA disinfectants were significantly more bactericidal against DSB of *S. aureus* than QT disinfectants. We also found that when DSB of *P. aeruginosa* were challenged with disinfectants, CL and HP were significantly more bactericidal than SH and QT disinfectants. Overall, we demonstrated that prolonged dehydration had varied effects on the bactericidal efficacy of disinfectants against DSB of *S. aureus* or *P. aeruginosa*. Specifically, we found that there were no significant differences in the bactericidal efficacies of disinfectants against 24 h and 72 h DSB of *S. aureus.* There was however, a significantly lower $\log_{10}$ reduction against 72 h DSB of *P. aeruginosa* compared to 24 h DSB of the same strain.

Bactericidal efficacy varies by strain after prolonged dehydration

Our study found differences in the overall bactericidal efficacy of disinfectants against DSB of *S. aureus* and *P. aeruginosa* after prolonged dehydration for 24 h and 72 h. While there was no significant difference in $\log_{10}$ reductions between 24 h and 72 h DSB of *S. aureus*, the reverse was true for DSB of *P. aeruginosa* as 72 h DSB of *P. aeruginosa* were harder to kill than their 24 h counterparts. In a previous study by our group, we found that 100% of *P. aeruginosa* DSB established at a dehydration temperature of 21°C were encased in EPS while this was true for only

92% of *S. aureus* DSB established at 25°C [34]. The consistent presence of EPS on DSB of *P. aeruginosa* at dehydration time points from 24 h to 120 h as previously demonstrated by our group suggested that older DSB of *P. aeruginosa* developed using our model may be encased in more EPS; making them harder to kill [34]. This is consistent with previous studies that have demonstrated the presence of a thick EPS matrix as a major factor for reduced bactericidal efficacy in biofilms compared to planktonic bacteria [29]. Moreover, previous studies [26, 37] have also suggested that unfavorable conditions such as dehydration may trigger bacterial biofilms to produce more EPS**.** While this may be true for *P. aeruginosa* DSB as evidenced in our previous study, the same may not be the case for *S. aureus* DSB as we found that older *S. aureus* DSB (72 h) were overall encased in less EPS matrix than 24 h biofilms [34]. More EPS production translates into a thicker barrier for disinfectants to bypass before contact with underlying bacteria. Additionally, a thicker EPS matrix may also result in a range of pH, which can impact bactericidal efficacy [18]. These factors could account for the reduced bactericidal efficacy against 72 h DSB of *P. aeruginosa* compared to 72 h DSB of *S. aureus.*

Product type and class significantly impact disinfectant efficacy against *S. aureus* DSB

There were significant differences among products, with QA1, QA2, CL, SH and HP1 being more bactericidal than QT. In a related study against *S. aureus* wet surface biofilms, Lineback et al., demonstrated that one sodium hypochlorite and five hydrogen peroxide disinfectants were significantly more bactericidal than two quaternary ammonium compounds [36]. This could be explained by the production of reactive oxygen species (ROS) by hydrogen peroxide disinfectants. The production of ROS results in more necrotic death compared to quaternary ammonium compounds as ROS result in DNA damage [38]. Comparatively, quaternary ammonium compounds mainly rely on a positively charged N-atom to bind to cell membranes, creating “pores” for n-alkyl side chains to transverse the cell membrane resulting in lysis and leakage of cytoplasmic contents [39, 40]. Considering the denser EPS produced by DSB compared to wet surface biofilms, this may present a significant barrier for quaternary ammonium products compared to sodium dichloro-s-triazinetrione, sodium hypochlorite and hydrogen peroxides. Moreover, oxidizing agents such as sodium dichloro-s-triazinetrione, sodium hypochlorite and hydrogen peroxides have low molecular weight active ingredients that when compared to larger molecules such as quaternary ammonium, can more easily bypass the cell membrane to damage internal cellular components [38]. This could further explain the observation that sodium dichloro-s-triazinetrione, sodium hypochlorite and hydrogen peroxide products were overall more bactericidal against DSB of *S. aureus* than quaternary ammonium. Quaternary alcohol products may have resulted in significantly higher bactericidal efficacies owing to the “rapid” bactericidal mode of action of alcohol [41].

We also found that the mean $\log_{10}$ reductions between HP1 and HP2; QA1 and QA2 were comparable when disinfectants were challenged with *S. aureus* DSB This finding is consistent with the findings of Lineback et al., 2018 who reported no significant differences among the bactericidal efficacies of five hydrogen peroxide products tested against *S. aureus* wet surface biofilms [36]. Similarly, in a recent study that evaluated the bactericidal efficacies of six disinfectant wipes against *S. aureus* ATTC-6538 inoculated on hard-non-porous surfaces, Voorn et al., reported no significant differences in the bactericidal efficacies among three hydrogen peroxide products or three quaternary alcohol products [42]. However, we found that quaternary alcohol products were overall more bactericidal than quaternary ammonium products without alcohol. This suggest that the defined percentage of alcohol added to quaternary ammonium compounds influences bactericidal efficacy; alcohol confers a rapid and more potent (tuberculocidal) action against bacteria [41].

HP and CL products are more bactericidal against *P. aeruginosa* DSB than SH, QT and QA products

Overall, CL, QA2, HP1 and HP2 had significantly higher $\log_{10}$ reductions against *P. aeruginosa* DSB than QA1, QT and SH. Our findings are similar to those of West et al., who demonstrated that hydrogen peroxide-based disinfectants are overall, more bactericidal against *P. aeruginosa* allowed to dry on a Formica disc than quaternary ammonium disinfectants [43]. In another study, Tote et al. found that hydrogen peroxides had a stronger antibiofilm activity against one day old *P. aeruginosa* biofilms as they were biologically active against both viable *P. aeruginosa* cells and their EPS matrix unlike isopropanol disinfectants [44]. The high efficacy of HP1 and HP2 compared to SH against DSB could be explained by the relatively low concentration (0.39%) of sodium hypochlorite in SH as in a 2018 study, Lineback et al. compared the bactericidal efficacies of 0.5% hydrogen peroxide and 1.312% sodium hypochlorite disinfectants against wet surface biofilms of *P. aeruginosa,* and found no difference in their efficacies [36]. The same intrinsic factor of a relatively low sodium hypochlorite concentration in SH may also account for the higher bactericidal efficacy of CL compared to SH as in a study by Tiwari et al., 0.60% sodium hypochlorite resulted in superior bactericidal efficacy against clinical isolates of *S. aureus* biofilms [45]. These reports suggest that although sodium hypochlorite is generally more bactericidal than quaternary ammoniums owing to their mode of action, the degree of disinfection is largely concentration dependent.

Although QA2 had a higher quaternary ammonium and lower alcohol content (0.76% quat + 22.5% alcohol) than QA1 (0.5% quat + 55% alcohol) (Table 1), QA2 demonstrated a significantly higher kill against *P. aeruginosa* DSB than QA1. This suggests that the synergistic effect of quaternary ammonium compounds and alcohol in QA1 may not be sufficient. Moreover, in a 2018 study by Wesgate et al., the authors reported that quaternary ammonium formulations with side alkyl chains in the $C_{12\text{-}16}$ range as is the case for QA1 were more adsorbed to different wipe material types than other formulations [46]. Consequently, and considering that wipes were “wringed” to dispense disinfectant liquid from QA1,

the quaternary ammonium compound in QA1 may have been more adsorbed to the wipe material than QA1, resulting in a lower final disinfectant liquid concentration in QA1 than QA2 [46].

P. aeruginosa DSB are harder to inactivate than *S. aureus* DSB

Our data delineate statistically significant higher average $\log_{10}$ reductions when disinfectants were treated against *S. aureus* DSB compared to *P. aeruginosa* DSB. Overall, the low bactericidal efficacy of disinfectants against biofilms is often linked to the EPS matrix [47]. The reduced efficacy of disinfectants, regardless of the product type, observed with Gram-negative *P. aeruginosa* can be partially explained by the presence of alginate, Psl, Pel [48], and extracellular DNA (eDNA) [49] as important components of the biofilm matrix characteristic of *P. aeruginosa*. Specifically, the overproduction of alginates by *P. aeruginosa* mutants result in the formation of larger microcolonies than wildtype strains [50]. This suggests a role for alginates in decreased susceptibility to antimicrobials [51] compared to non-alginate-producing bacteria such as *S. aureus* [48]. Pel, on the other hand, plays a vital role in cell-to-cell interactions within these biofilms [52] and in the biofilm maturation [49]. A spike in alginate and carbohydrate production during biofilm formation and maturation confers an overall increase in the net negative charge of the EPS matrix, enhancing the electrostatic attractions between the EPS matrix and positively charged antimicrobials as quaternary ammonium compounds [47]. This limits the diffusion of cationic antimicrobials through the EPS matrix, thus shielding the underlying bacteria from direct antimicrobial contact [47]. However, the cell wall of Gram-positive bacteria such as *S. aureus* is essentially composed of peptidoglycan and teichoic acid and substances with high molecular weight can traverse the cell wall. [53]. This may explain the higher $\log_{10}$ reductions observed against *S. aureus* DSB compared to *P. aeruginosa* DSB exposed to quaternary alcohol and quaternary ammonium products.

Our results suggest that comparatively higher mean $\log_{10}$ reductions are achieved when sodium hypochlorite was challenged with *S. aureus* compared to *P. aeruginosa* DSB. This could be due to the act that negatively charged disinfectants as sodium hypochlorite destroy the cellular activity of bacterial proteins [54] and are capable of increased penetration of outer cell layers even in unionized state [53]. Similarly, hydroxyl free radicals from HP based products specifically target sulfhydryl groups, double bonds [55] and destroy bacterial lipids, proteins, and DNA. Our data is in accordance with Lineback et al., 2018 who suggested that sodium hypochlorite products are overall, more effective against *P. aeruginosa* and *S. aureus* WSB compared to quaternary ammonium products [36].

Our results support previous findings that DSB are harder to kill than planktonic bacteria; all the products tested in this study are EPA registered, indicating high levels of efficacy against planktonic bacteria of *S. aureus* and *P. aeruginosa.* To reduce patient safety risks in healthcare facilities, it is critical to conduct baseline disinfectant efficacy testing for product registration using bacteria biofilms representing healthcare environments.

We acknowledge that the scope of our study is limited as we did not investigate the bactericidal efficacy of the tested products against mixed culture bacterial biofilms common on dry contaminated hard-non-porous surfaces in healthcare facilities. We also acknowledge that our study did not specifically investigate disinfectant efficacy against DSB of *S. aureus* and *P. aeruginosa* subjected to longer hours of dehydration as this could impact the efficacy levels of commonly used disinfectants. A wider range of disinfectant active ingredients could have also been investigated. However, this study has set the foundation for future investigations of DSB of *S. aureus* and *P. aeruginosa*.

Conclusion

Although it is generally agreed that DSB pose a severe challenge for the disinfection of hard non-porous surfaces in healthcare facilities and are a significant contributor to the incidence of HAI, the success of any disinfection regime is dependent on multiple intrinsic and extrinsic factors. Our study definitively demonstrated that significant kill levels of the DSB of major healthcare pathogens that cause HAI can be achieved although this is highly dependent on the choice of disinfectant, active ingredient class, DSB “age” and bacteria strain. It is therefore critical for healthcare stakeholders to consider these factors in efforts to reduce HAI rates.

Abbreviations

ATCC: American type Culture collection

CDC: Center for Disease Control

DSB: Dry surface biofilms

EPA: Environmental Protection Agency

EPS: Extracellular polymeric substances

HAI: Healthcare-associated infection

PBS: Phosphate buffered saline

WSB: Wet surface biofilms

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: All quantitative data generated or analysed during this study are included in this published article.

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Authors' contributions: CAN and GKC conducted the wet lab procedures, analysed and interpreted the data generated, and wrote the manuscript. XL provided industry experience, designed elements of the experimental protocol, and was a contributor in writing and editing the manuscript. PT also provided industry experience and was a contributor in writing and editing the manuscript. HFO served as the principal investigator for the study and was a contributor in writing and editing the manuscript. All authors read and approved the final manuscript.

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Figures

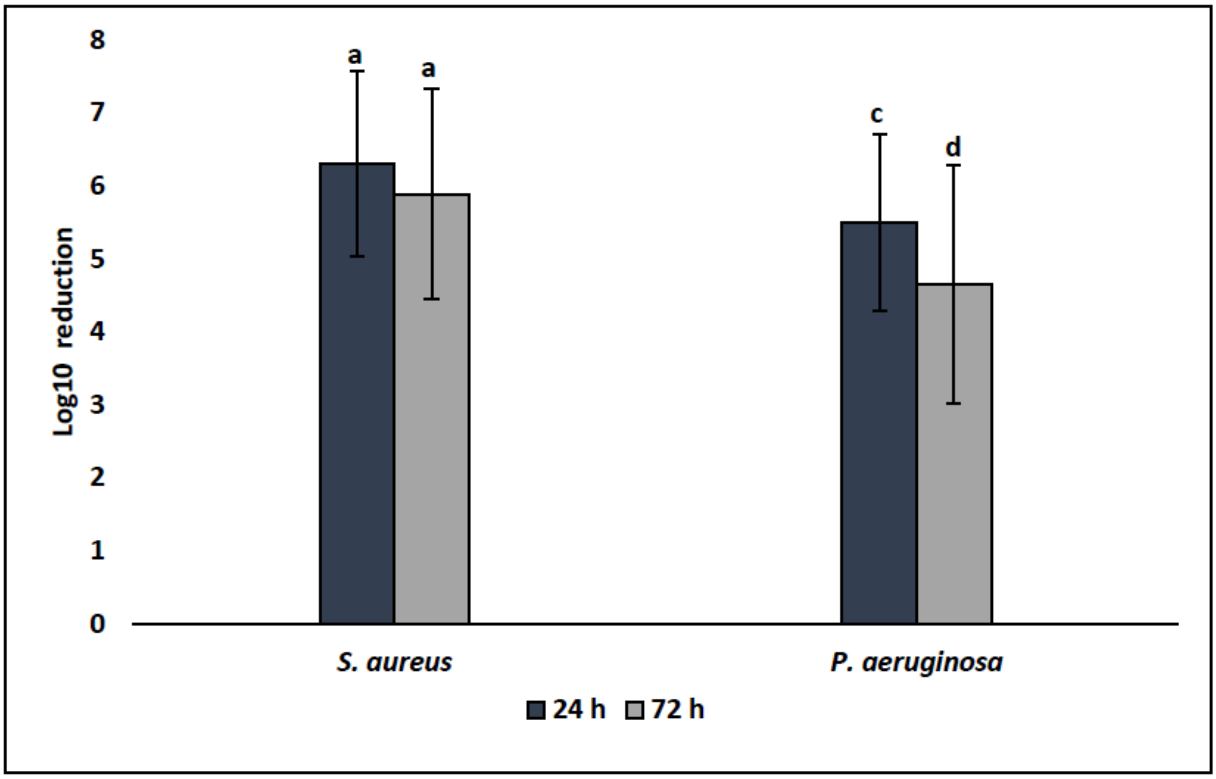

Figure 1

Disinfectant efficacy against DSB of S. aureus and P. aeruginosa by dry time. Letter (a) Tukey grouping for disinfectant efficacy against S. aureus DSB only. Letters are (c, d) Tukey grouping for disinfectant efficacy against DSB of P. aeruginosa only.

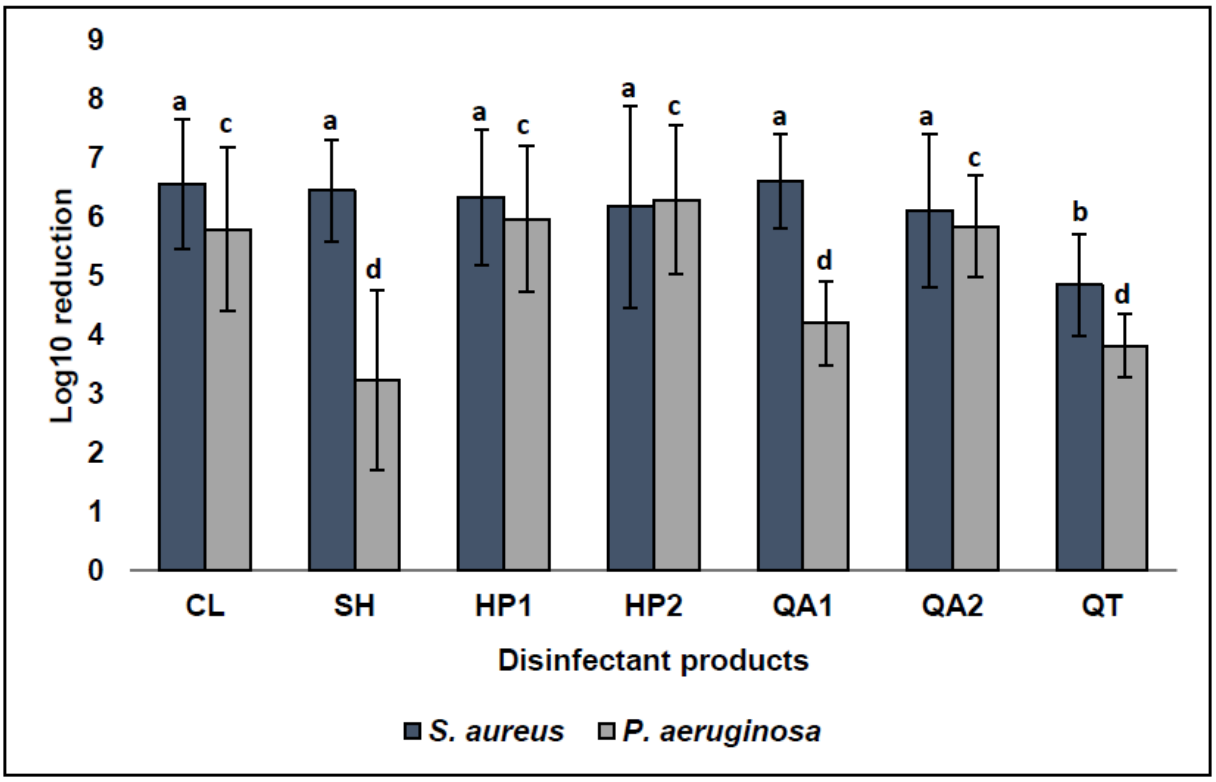

Figure 2

Disinfectant efficacy against DSB of S. aureus and P. aeruginosa by product type. Letters (a, b) Tukey grouping for disinfectant efficacy against S. aureus DSB only. Letters are (c, d) Tukey grouping for disinfectant efficacy against DSB of P. aeruginosa only.

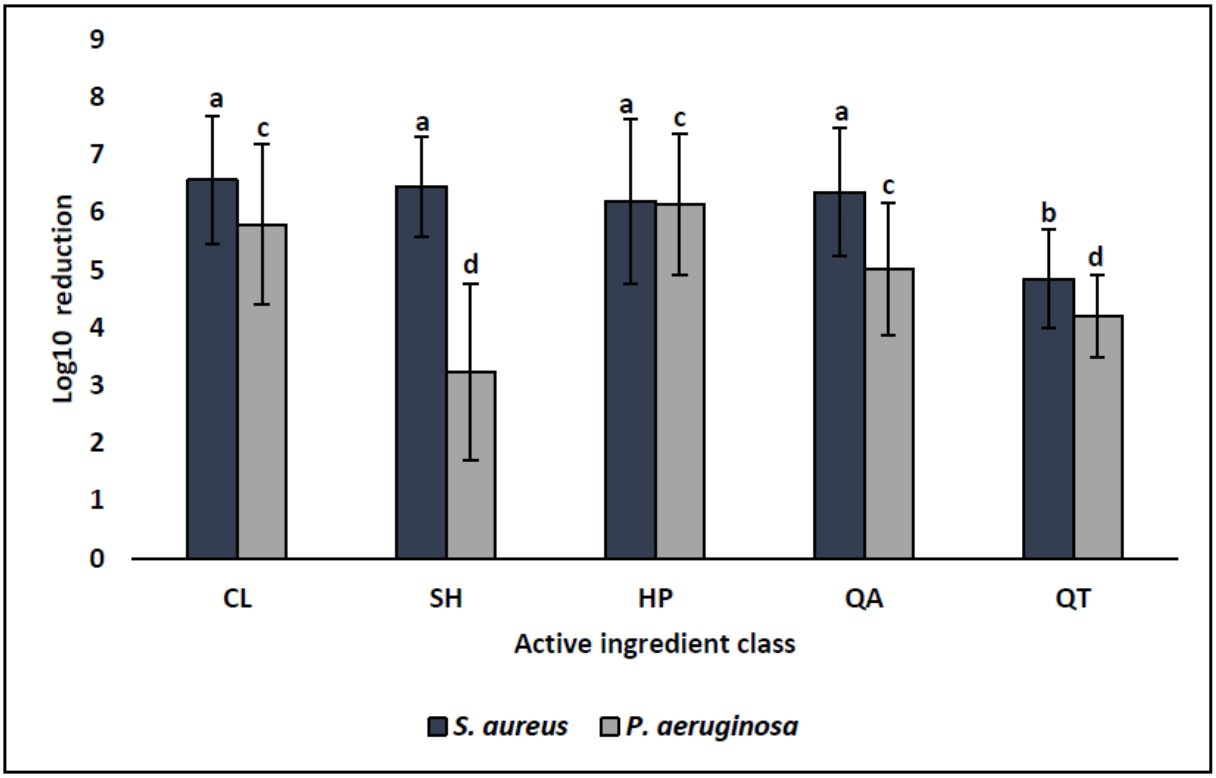

Figure 3

Disinfectant efficacy against DSB of S. aureus and P. aeruginosa by active ingredient class. Letters are (a, b) Tukey grouping for disinfectant efficacy against S. aureus DSB only. Letters are (c, d) Tukey grouping for disinfectant efficacy against DSB of P. aeruginosa only.