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ORIGINAL ARTICLE

Dual species dry surface biofilms; *Bacillus* species impact on *Staphylococcus aureus* survival and surface disinfection

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Abstract

Aims: Dry surface biofilms (DSB) survive on environmental surfaces throughout hospitals, able to resist cleaning and disinfection interventions. This study aimed to produce a dual species DSB and explore the ability of commercially available wipe products to eliminate pathogens within a dual species DSB and prevent their transfer.

Methods and Results: *Staphylococcus aureus* was grown with two different species of *Bacillus* on stainless steel discs, over 12 days using sequential hydration and dehydration phases. A modified version of ASTM 2967–15 was used to test six wipe products including one water control with the Fitaflex Wiperator. *Staphylococcus aureus* growth was inhibited when combined with *Bacillus subtilis*. Recovery of *S. aureus* on agar from a dual DSB was not always consistent. Our results did not provide evidence that *Bacillus licheniformis* protected *S. aureus* from wipe action. There was no significant difference of *S. aureus* elimination by antimicrobial wipes between single and dual species DSB. *B. licheniformis* was easily transferred by the wipe itself and to new surfaces both in a single and dual species DSB, whilst several wipe products inhibited the transfer of *S. aureus* from wipe. However, *S. aureus* direct transfer to new surfaces was not inhibited post-wiping.

Conclusions: Although we observed that the dual DSB did not confer protection of *S. aureus*, we demonstrated that environmental species can persist on surfaces after disinfection treatment. Industries should test DSB against future products and hospitals should consider carefully the products they choose.

Significance and Impact of the Study: To our knowledge, this is the first study reporting on the production of a dual species DSB. Multispecies DSB have been identified throughout the world on hospital surfaces, but many studies focus on single species biofilms. This study has shown that DSB behave differently to hydrated biofilms.

KEYWORDS

Bacillus, biocides, biofilms, disinfection, staphylococci

INTRODUCTION

Biofilms are considered the most prevalent form of microbial existence in natural ecosystems (Nozhevnikova et al., 2015). These complex communities of microorganisms are embedded into an extracellular matrix, which aids in their protection from external stressors such as antimicrobials (Francolini & Donelli, 2010). Multispecies biofilms have a different spatial organization compared to monospecies, which contributes to the fitness of the whole population within the biofilm (Elias & Banin, 2012). Interactions between the different species within a biofilm can change the structural and functional dynamics, influencing pathogenicity of the biofilm and promoting antimicrobial resistance (Harriott & Noverr, 2010).

The majority of in vitro studies predominantly focus on single species biofilms, most likely due to experimental limitations with complex communities of organisms (Hall-Stoodley et al., 2004; Sanchez-Vizuete et al., 2015). However, this is not representative of what occurs in most real-world scenarios, where biofilms comprise of multiple species, both pathogenic and not. This is widely evidenced in biofilms residing on ships hulls, in wastewater treatment, oral cavities, medical devices and wounds for example (Vishwakarma, 2020).

The global burden of healthcare associated infections (HCAIs) puts huge economic strain on healthcare providers. For example, NHS England spends an average of £2.1 billion per year for the treatment of HCAIs (Guest et al., 2020). Medical equipment such as urinary catheters (Wilks et al., 2021) present suitable surface material for the attachment of bacteria leading to biofilm development and consequently HCAIs. Endoscopes are also commonly associated with biofilms even after the guideline disinfection of the equipment (Bisset et al., 2006; Johani et al., 2018). In clinical settings, around 65% of HCAIs are caused by hydrated biofilms (Percival et al., 2015).

Several studies investigating the tolerance of multispecies biofilms to biocides, including chlorine (Schwering et al., 2013) and benzalkonium chloride (Ibusquiza et al., 2011), observed a decrease in biocide susceptibility of multispecies biofilms compared to monospecies biofilms (Sanchez-Vizuete et al., 2015). Resident flora, for example non-pathogenic bacteria found within the oral cavity, can protect pathogenic species from disinfection (Luppens et al., 2008). Bridier et al. (2012) evidenced the protection of a pathogenic strain of *Staphylococcus aureus* from peracetic acid disinfection when embedded in a hydrated biofilm of a non-pathogenic *Bacillus subtilis*, isolated from an automated endoscope washer disinfector (AEWD).

Surface cleaning and disinfection is important in the reduction and control of pathogen transmission and survival in the environment as we know that many common nosocomial pathogens, including *S. aureus*, can survive for up 7 months on inanimate surfaces (Kramer et al., 2006). Cleaning and disinfection further help reduce HCAIs (Gebel et al., 2013). However, microbial biofilms are also present in the environment on surfaces in a dry state that often go unnoticed and have been termed "visually clean" (Vickery et al., 2012). These dry surface biofilms (DSB) have received limited attention with regards to biofilm resistance to disinfectants and other antimicrobial compounds, and the effectiveness of cleaning/disinfection measure used in healthcare. DSB are biofilms that have been exposed to lowered water potential, reduced nutrient resources and periodic cleaning/disinfection of clinical surfaces (Almatroudi et al., 2015; Ledwoch et al., 2019). The presence of DSB with very thick exopolymeric substance (EPS) was confirmed by Hu et al. (2015). This thick EPS layer creates a protective barrier to desiccation and increases tolerance to cleaning and disinfection regimens, ultimately contributing to a prolonged microbial survival on healthcare surfaces (Hu et al., 2015). Environmentally isolated DSB are polymicrobial, containing both pathogenic and non-pathogenic species (Ledwoch et al., 2018). We hypothesise that non-pathogenic *Bacillus* spp. will protect *S. aureus*, a nosocomial pathogen, from the biocidal action of wipe products.

This study is the first to explore the production of dual species DSB combining a non-pathogenic species with a pathogen, and to explore the potential impact dual species DSB have on the efficacy of commonly used disinfectant product.

MATERIALS AND METHODS

Bacterial growth

B. subtilis washer-disinfector isolate (AEWD) (Martin et al., 2008), *B. licheniformis* ATCC 14580 and *S. aureus* NCTC 10788 were used. Whilst the *B. subtilis* isolate is a heavy biofilm producer (Martin et al., 2008), *B. licheniformis* is a predominant species isolated from DSB in healthcare settings (Ledwoch et al., 2018). Overnight cultures of each species were prepared in 20 ml^{-1} of sterile tryptone soya broth (TSB) and grown aerobically at 37°C at 120 rpm in a shaking incubator (Sanyo Orbital Shaker).

Dry surface biofilm formation

DSB were grown on sterile stainless steel discs AISI 430 (0.7 ±0.07 mm thickness; 10 ±0.5 mm diameter) placed in a Corning Costar flat bottom 24 well cell culture plate,

following methods outlined by Ledwoch et al. (2019). DSB were formed over a 12-day period, alternating between 48 h cycles of hydration and dehydration lead to the production of a mature DSB (Ledwoch et al., 2019). For single species DSB, 1 ml^{-1} inoculum (10^6 CFU ml^{-1}) with 3 gl^{-1} of bovine serum albumin (BSA) was dispensed into wells at the initial hydration phase. Well plates were incubated at room temperature (20–23°C) for 48 h, with continuous shaking at 200 rpm. Media was then drained out of each well and incubated for 48 h at 37°C. This process was repeated until the 12 days were complete. Each hydration phase included the addition of 1 ml^{-1} of TSB and BSA at 3 gl^{-1}.

For dual species DSB, bacterial inoculum consisting of 10^6 CFU ml^{-1} of either *B. licheniformis* or *B. subtilis* and 10^{6-7} CFU ml^{-1} of *S. aureus* with 3 gl^{-1} BSA was used as the start-up inoculum. The ratio (0.5:1) was found to produce the most consistent dual species DSB (data not shown). The overnight washed broth culture of *Bacillus* spp. used as an inoculum was not checked for the presence of endospores, since the overnight broth culture conditions were optimal for bacterial growth and not conducive for sporulation.

Product testing

Six different commercially available wipes (Table 1) were investigated. All wipes were pre-packaged excluding the control (water) wipe.

Wipe efficacy was tested using the Wiperator (FitaFlex Ltd., Ontario, Canada) as described in the ASTM2967-15 standard (ASTM 2967–15, 2015). Discs of single or dual DSB were wiped for 10 s at 500 g pressure, left for 60 seconds post-wiping before being placed in a neutralizer (neutralizing solution: 8.5 gl^{-1} sodium chloride, 1 gl^{-1} tryptone, 1 gl^{-1} L-Histidine, 3 gl^{-1} lecithin, 5 gl^{-1} sodium thiosulphate, 30 gl^{-1} polysorbate 80 and 30 gl^{-1} saponin) for 10 min. Neutralizer toxicity tests were completed prior to experimentation (data not shown).

TABLE 1 Commercial wipe products used and control wipe

Product	Formulation/active ingredients
Control (water)	Water on Rubber Rubbermaid® HYGEN™ disposable microfiber cloth 284
A	>5% non-ionic surfactants and preservatives
B	DDAC (6.4 g/Kg) N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine (5.31 g/Kg)
C	DDAC 0.450% w/w
D	<1% cationic biocides Additional surfactants
E	Peracetic acid

Determination of Log_{10} reduction within the DSB for each species

The effectiveness of each wipe was first determined by the reduction in bacterial viability, expressed as log_{10} reduction in CFU ml^{-1}. Following wiping and neutralization, the discs were placed in 2 ml^{-1} of TSB with 1 g of borosilicate glass beads and incubated at 37°C for 2 hrs, to allow bacteria to become metabolically active without growing. This step has been shown to be essential for the recovery of bacteria from DSB (unpublished data). After incubation samples were vortexed for 4 min using the multitube vortexer (FisherBrand), serially diluted to 10^7 and 200 μl^{-1} of each dilution was spread onto a TSA plate. Log_{10} reduction was calculated as the difference in bacterial number between wiped samples and unwiped DSB. Specific selective media to distinguish between *Bacillus* spp. and *S. aureus* were not used to avoid introduction of potential additional stressors post wiping (data not shown). Instead, TSA plates were used since *S. aureus* was easily distinguishable against *Bacillus* spp. due to the characteristic phenotypes of the colonies. Whilst *S. aureus* produces small round colonies that are golden yellow in colour, *Bacillus* spp. produce larger colonies that are whitish in colour.

Direct wipe transfer of bacteria

The transferability of bacteria to a new surface, mimicking what may occur within hospitals following routine cleaning and disinfection regimens, was investigated. The DSB disc was wiped as described above and a sterile disc was wiped immediately using the same wipe as instructed in the ASTM2967-15 protocol (ASTM 2967–15, 2015). This new disc was then placed in neutralizer for 10 min, and the resuscitation of bacteria and bacterial count was performed as described above.

Transfer of bacteria following wiping

Another transfer test was conducted to measure the bacterial transferability from the wiped discs. Following wiping, each disc was pressed 36 times at 100 g pressure onto a Dey Engley (DE) neutralizing agar plate (Ledwoch et al., 2019). Each plate was incubated at 37°C overnight. Positive growth was recorded, and transferability was calculated as the number of positive contacts out of the 36 adpressions and expressed as percentage transfer.

Scanning electron microscopy

High vacuum SEM was conducted to visualize the structure and characteristics of the dual DSB. DSB samples were prepared by overnight incubation of discs in 2.5% glutaraldehyde solution, followed by a series of ethanol washes; 5%, 25%, 50%, 75%, 90% and 100% ethanol for 10 min in each concentration to fix the samples to the surface of the disc. DSB were then coated with a thin layer (20 nm) of gold–palladium using a Bio-Rad Sputter Coater SC500. Argon gas was used to purge the sputter chamber before coating. Images were taken using the InLens setting on a Philips XL30 field emission gun-scanning electron microscope (FEG-SEM) at ×10,000 and ×5000 magnification and 5–7 mm working distance.

Low vacuum SEM imaging was used to identify whether or not bacteria from the DSB were retained within the wipe following treatment. Only wipes A–E (detergent/disinfectant wipes) were analysed. Discs containing dual DSB were wiped on the same day as imaging took place. Used wipes were cut to 10x10mm squares and immediately attached to 12.5 mm aluminium SEM stubs (TAAB Laboratories Equipment Ltd) without any treatment and imaged with a Tescan MAIA3 FEG-SEM at a working distance of 5 mm. Samples were analysed at −5 °C at 50 or 80 Pa.

Statistical analysis

Statistical difference between data sets was calculated using GraphPad PRISM® (version 9.3.1, GraphPad Software Inc.) using two-way analysis of variance (ANOVA). Post hoc tests, and Dunnett's and Sidak's multiple comparison test were also used. A minimum of three biological replicates were performed for each experiment. Standard deviation was also calculated in GraphPad.

RESULTS

The environmental isolate, *B. subtilis* AEWD inhibited the growth of *S. aureus* when grown as a dual DSB (Table 2). The formation of a dried dual *S.* aureus/*B. licheniformis* species biofilm was confirmed with SEM imaging (Figure 1). Whilst both bacterial species can be clearly identified, *S. aureus* culturability when the DSB was plated on TSA was not always consistent as *S. aureus* only grew in 50% of samples following DSB formation (Table 2).

Although inconsistencies in culturability of *S. aureus* within the dual DSB were identified, repeats where there was positive growth of *S. aureus* in the untreated control were selected to allow for a better understanding of the protection of *S. aureus* by *B. licheniformis*. Wipes B–E performed better against dual species DSB than single species DSB (Figure 2). Interestingly, wipe B had little activity against *S. aureus* single species DSB (average 1.6 $\log_{10}$ reduction) compared to dual species DSB (average 3.7 $\log_{10}$ reduction). Overall wipes A and C–E performed best against both single and dual species DSB (Figure 2). Variability in results observed with some products with the dual DSB originated with some low *S. aureus* count in dual DSB. Of note, *S. aureus* results from the single DSB were consistent with what has been previously reported in other studies (Almatroudi et al., 2018; Ledwoch et al., 2019). Overall, the efficacy of all products tested against the *S. aureus* single DSB was not statistically different than the water control (Dunnett's multiple comparison test, $p > 0.05$) except for wipe B (Dunnett's multiple comparison test, $p < 0.05$). There was no statistically significant difference (one-way ANOVA, $p > 0.05$) between the water control and wipe products A–E when *S. aureus* was in a dual DSB.

The majority of commercially available disinfectant and detergent wipes used here, did not effectively eradicate

TABLE 2 Culturability of both bacterial species from DSB recovered on TSA plates. Starting inocula: *Bacillus licheniformis*: 6 Log_{10} CFU ml^{-1}; *B. subtilis*: 6 Log_{10} CFU ml^{-1}; *Staphylococcus aureus*: 6 Log_{10} CFU ml^{-1}.

	Log_{10} CFU ml^{-1} recovered after DSB formation			
	***B. licheniformis–S. aureus* dual DSB**		***B. subtilis–S. aureus* dual DSB**	
Biological repeats	***B. licheniformis***	***S. aureus***	***B. subtilis***	***S. aureus***
1	5.6	5.04	5.5	No growth
2	5.8	5.84	5.5	No growth
3	6.0	No growth	5.9	No growth
4	5.2	No growth	6.2	No growth
5	5.4	4.0	6.3	No growth
6	5.6	No growth	—	—

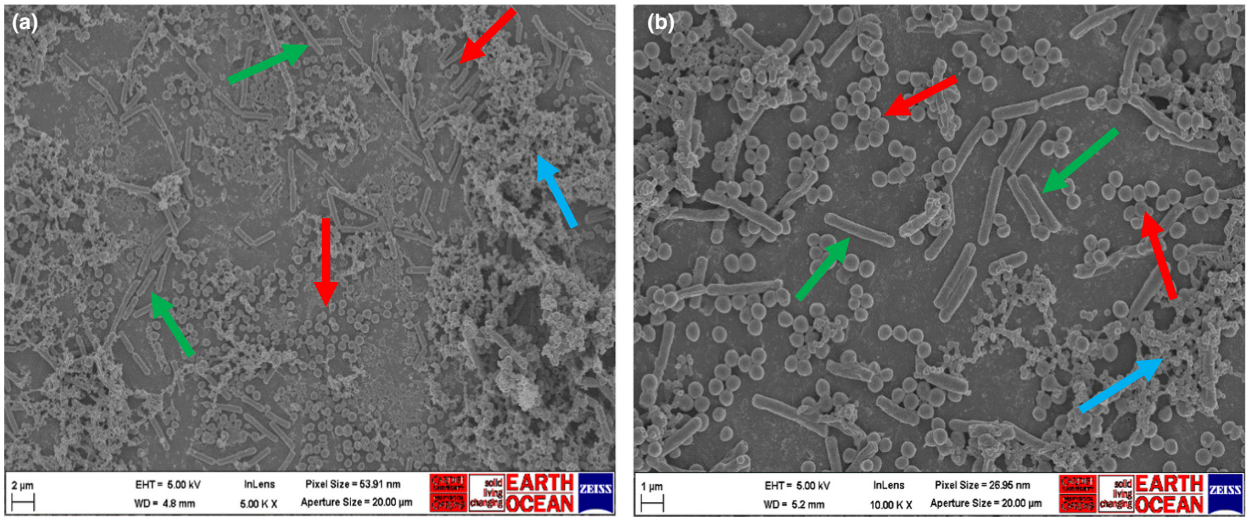

FIGURE 1 Scanning electron microscope imaging of dual dry surface biofilms (*Bacillus licheniformis* and *Staphylococcus aureus*) at (a) ×5000 magnification and (b) ×10,000 magnification. Both bacterial species can be identified (green arrows: *B. licheniformis*; red arrows: *S. aureus*), together with the presence of EPS (blue arrows). To note, there was no evidence of bacterial endospores. Images presented are representative of the whole disc surface. Observations were made on three independent repeats

B. licheniformis in DSB (Figure 3). In single species DSB, *B. licheniformis* seemed to be more resistant to cleaning and disinfection than in dual species DSB, although differences were not statistically significant (two-way ANOVA, $p > 0.05$). There was no statistically significant difference in activity between the water control and any of the products tested (one-way ANOVA, $p = 0.21$) against *B. licheniformis* single DSB (Figure 3). Wipe E performed the best overall when considering dual species DSB with a mean $\log_{10}$ reduction of 3.5 (Figure 3), although there was no statistical difference in bacterial reduction between the water control and any of the wipes tested (Dunnett's multiple comparison test, $p > 0.05$). The variability in results with the DSB likely impacted the statistical difference significance, as one biological repeat had very low untreated control counts of *B. licheniformis* leading to negative $\log_{10}$ reduction values (Figure 3).

The efficacy of antimicrobial wipes is measured by the number of bacteria removed/killed on surfaces but also by the ability of the wipe not to transfer microorganisms to other surfaces (Wesgate et al., 2019). Wipe products (B-E) did not transfer any *S. aureus*, regardless of being in a single or dual species DSB (Table 3). The water control wipes transferred significantly (two-way ANOVA, $p < 0.05$) more *S. aureus* (>4 $\log_{10}$) in a single species DSB, than any of the wipe products (Table 3). More *S. aureus* were transferred from the single species biofilms (circa 3–4 $\log_{10}$) than from the dual biofilm (circa 1–2 $\log_{10}$) (Table 3). There was a statistically significant difference between $\log_{10}$ transfer of *S. aureus* in a single DSB versus dual DSB for both water control and wipe A (Sidak's multiple comparison test, $p < 0.05$).

Overall, wipe products including water control transferred high concentrations of *B. licheniformis* (2–4.6 $\log_{10}$ transfer) following wiping of DSB both in a single and dual species DSB. There was a statistically significant difference in wipe transfer of *B. licheniformis* between the dual and single DSB for all products (two-way ANOVA, $p < 0.05$) (Table 4). Sidak's multiple comparison test revealed wipe E had the greatest significant difference in *B. licheniformis* transfer from wipes between dual and single DSB ($p = 0.0005$).

We also investigated whether any viable bacteria in DSB remaining on surfaces post-wiping could be directly transferred. Results from dual species DSB displayed a greater overall variability in data than single species, as shown in Figures 4 and 5. Overall, *B. licheniformis* remaining on surfaces post wiping was easily transferred by the direct adpression of the surface to DE agar (Figure 4). All wipes transferred >90% of *B. licheniformis* from dual species DSB except for wipe A. All wipes transferred 100% of *B. licheniformis* from single species DSB (Figure 4). With *S. aureus*, all wipes transferred >90% *S. aureus* from single DSB. *S. aureus* direct transfer was much reduced (<10%) from dual species DSB following wiping with products D and E (Figure 5). Variation was highest with wipes B–E, when considering percentage transfer of *S. aureus* from dual DSB, Figure 5 shows the range of data from 0%–100%.

Following wiping of dual species DSB disc, wipe materials were imaged under low vacuum SEM. The presence of DSB within all wipes is evident from imaging, with bacterial clusters present in wipe fibres (Figure 6). Aggregates of DSB are mainly seen in wipes A–D; however, images of wipes E show a homogenous spread of bacteria all over

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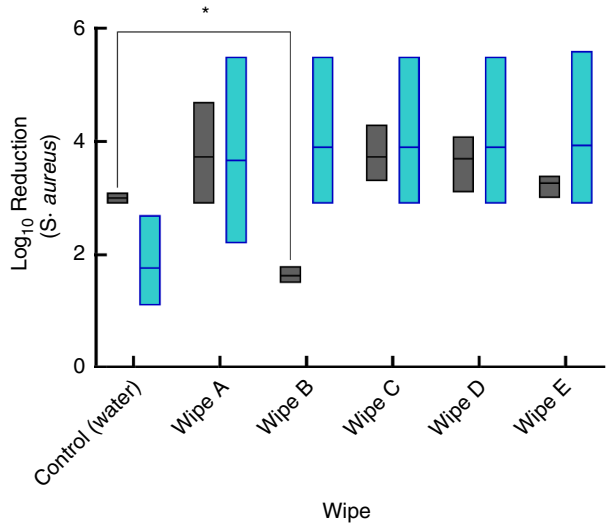

FIGURE 2 Product efficacy against *Staphylococcus aureus* in a single or dual species DSB after 60 s time post wiping. Single spp. DSB (■), dual spp. DSB (■). There was no statistically significant difference between the single and dual species DSB (two-way ANOVA, $p > 0.05$). There was no statistically significant difference between the water control and products a, C, D and E used when *S. aureus* was in a single or dual DSB (one-way ANOVA, $p > 0.05$). There was a statistical difference between the water control vs. wipe B when *S. aureus* was embedded in a single species DSB (Dunnett's multiple comparison test, $p < 0.05$). Bars represent range of $\log_{10}$ reduction values, with the mean outlined by the line in the middle of the bar

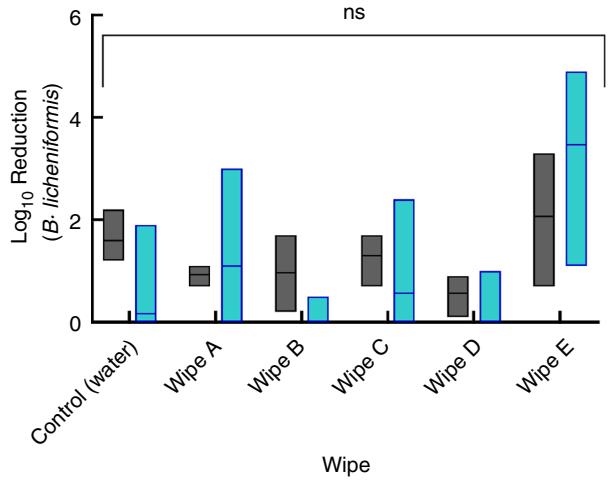

FIGURE 3 Product efficacy against *Bacillus licheniformis* in a single or dual species DSB after 60 s time post wiping. Single spp. DSB (■), dual spp. DSB (■). There was no statistically significant difference between the single and dual DSB from all wipes (two-way ANOVA, $p > 0.05$). No significant (ns) difference was identified between the water control and each wipe product for both single and dual species DSB (Dunnett's multiple comparison test, $p > 0.05$). Bars represent range of $\log_{10}$ reduction values, with the mean outlined by the line in the middle of the bar

TABLE 3 $\log_{10}$ transfer of *Staphylococcus aureus* from single and dual species DSB at 60 s contact time. Values indicate mean $\log_{10}$ CFU transferred with ±standard deviation (SD). Statistical analysis was performed comparing single and dual species DSB.

	$\log_{10}$ transfer of bacteria (±SD)	
Wipe	**Single species DSB**	**Dual species DSB**
Water (control)	4.1 (±0.3)*	2.0 (±1.7)
A	3.2 (±0.2)*	0.9 (±0.3)
B	No transfer	No transfer
C	No transfer	No transfer
D	No transfer	No transfer
E	No transfer	No transfer

*$p < 0.05$.

TABLE 4 $\log_{10}$ transfer of *Bacillus licheniformis* from single and dual species DSB at 60 s contact time. Values indicate mean $\log_{10}$ CFU transferred with ±standard deviation (SD). Statistical analysis was performed comparing single and dual species DSB.

	$\log_{10}$ transfer of bacteria (±SD)	
Wipe	**Single species DSB**	**Dual species DSB**
Water (control)	3.9 (±0.6)*	2.3 (±0.5)
A	4.1 (±0.5)*	2.8 (±0.6)
B	4.6 (±0.4)*	3.1 (±1.0)
C	4.6 (±0.2)*	3.4 (±0.1)
D	4.6 (±0.1)*	3.2 (±0.2)
E	3.6 (±0.3)*	1.7 (±0.5)

*$p < 0.05$.

the wipe material. Wipe E has a much rougher texture than the other wipes, where the fibres are smooth (data not shown).

DISCUSSION

Approximately 40–80% of all living bacteria on earth reside in the form of a biofilm (Hall-Stoodley et al., 2004). The complexity and distinct spatial organization of multispecies biofilms presents advantages in natural habits compared to monospecies biofilm (Røder et al., 2020), including an increased tolerance to disinfectants such as chlorine (Schwering et al., 2013).

DSB have been shown to be prominent throughout hospitals, colonizing various dry surfaces (Hu et al., 2015; Ledwoch et al., 2018). Dry surfaces greatly contribute to

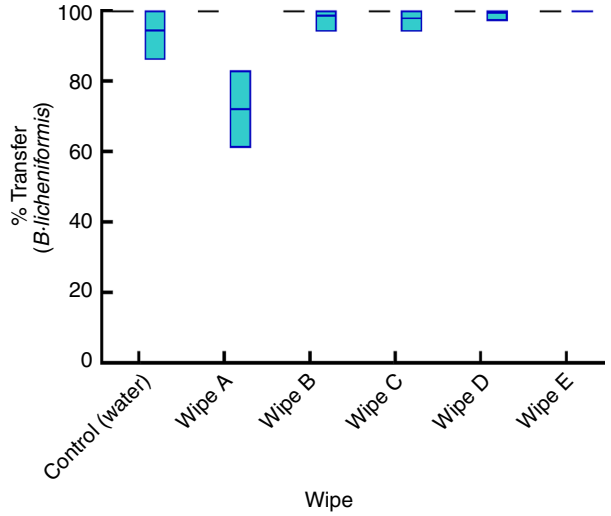

FIGURE 4 Percentage of direct transfer of *Bacillus licheniformis* from single and dual species DSB. Single spp. DSB (■), dual spp. DSB (■). Direct transfer was measured after pressing disc directly onto DE agar following wiping. Percentage transfer was calculated as the number of squares containing growth divided by the total squares (36) multiplied by 100. Bars represent range of $\log_{10}$ reduction values, with the mean outlined by the line in the middle of the bar

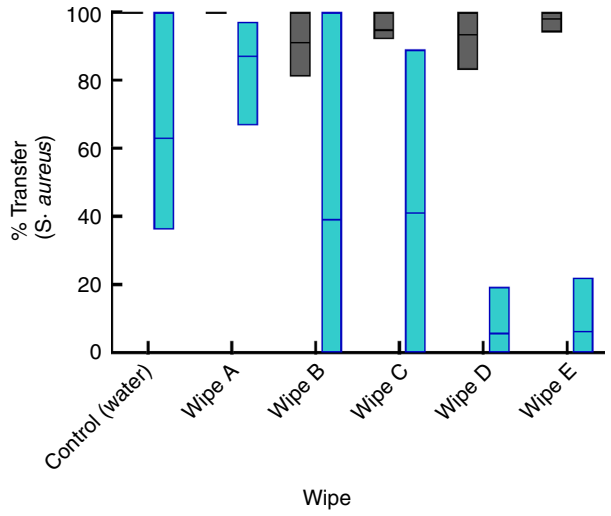

FIGURE 5 Percentage of direct transfer of *Staphylococcus aureus* from single and dual species DSB. Single spp. DSB (■), dual spp. DSB (■). Direct transfer was measured after pressing directly onto DE agar following wiping. Percentage transfer was calculated as the number of squares containing growth divided by the total squares (36) multiplied by 100. Bars represent range of $\log_{10}$ reduction values, with the mean outlined by the line in the middle of the bar

the contamination of near patient environments and provide ideal substrate for microbial contamination over prolonged periods of time (Weber et al., 2013). Cleaning and disinfection of these dry surfaces is essential for patient recovery and reduction in HCAIs (Doll et al., 2018).

To our knowledge, this is the first study to produce a dual species dry surface biofilm for testing against commercially available wipes. We measured the efficacy of commercially available wipe products using three different parameters: reduction in bacterial viability, direct surface to surface transfer post wiping and transfer of bacteria by the wipe itself.

It is important to establish the difference between the terms viability and culturability when discussing the results presented here. Bacterial viability has been defined as the capacity for replication or the ability to grow, whereas culturability is the detectable replication of bacteria, that is growth on agar (Barer & Harwood, 1999). We observed a large variation in culturability of *S. aureus* on agar when in a dual DSB with *B. licheniformis*. Behaviour between species in a biofilm can be cooperative, competitive or neutral (Alonso et al., 2020; Li et al., 2021; Nadell et al., 2016). Cooperation can include protection from biocides whereas competition may be the result of lack of space, nutrients and other resources (Alonso et al., 2020). Results inconsistency could be attributed to competition between the two species, *B. licheniformis* becomes the dominant species and thus *S. aureus* is hard to identify on agar. Gause's law states that two species in competition cannot remain at stable levels when exposed to limited resources over time, so one must become dominant. The same principle is observed with *B. subtilis* and *S. aureus*. The observed inhibitory effect of *B. subtilis* against *S. aureus* supports evidence of its use as a probiotic cleaner, as it has been shown to counteract growth of pathogens on dry hospital surfaces (Caselli et al., 2016). Microbial cleaning with *B. subtilis* on hospital surfaces has been shown to be effective in reducing HCAIs over prolonged periods compared to more conventional disinfectant cleaning methods (Vandini et al., 2014).

In our original hypothesis, we stated that non-pathogenic environmental *Bacillus* could protect a pathogenic species, such as *S. aureus* from disinfection. Bridier et al. (2012) demonstrated the impact of *B. subtilis* hydrated biofilm to protect *S. aureus* against 3500 ppm peracetic acid. Such a protective effect was not observed in our study. Instead, *B. subtilis* AEWD (the same isolate used in Bridier's study) inhibited the growth of *S. aureus*. When *B. licheniformis* was used in the dual DSB, no protective effect was observed (Figures 2 and 5; Table 3). A noticeable difference between hydrated biofilm and dual species DSB was the amount of EPS observed in the biofilm. Although our SEM images indicated the presence of EPS in the DSB, which is consistent with other SEM images of DSB (Ledwoch et al., 2019), the extensive EPS network, confirmed through congo red staining, described by Bridier et al. (2012) in hydrated biofilm of *B. subtilis* AEWD was not observed here with dual species DSB. The impact of

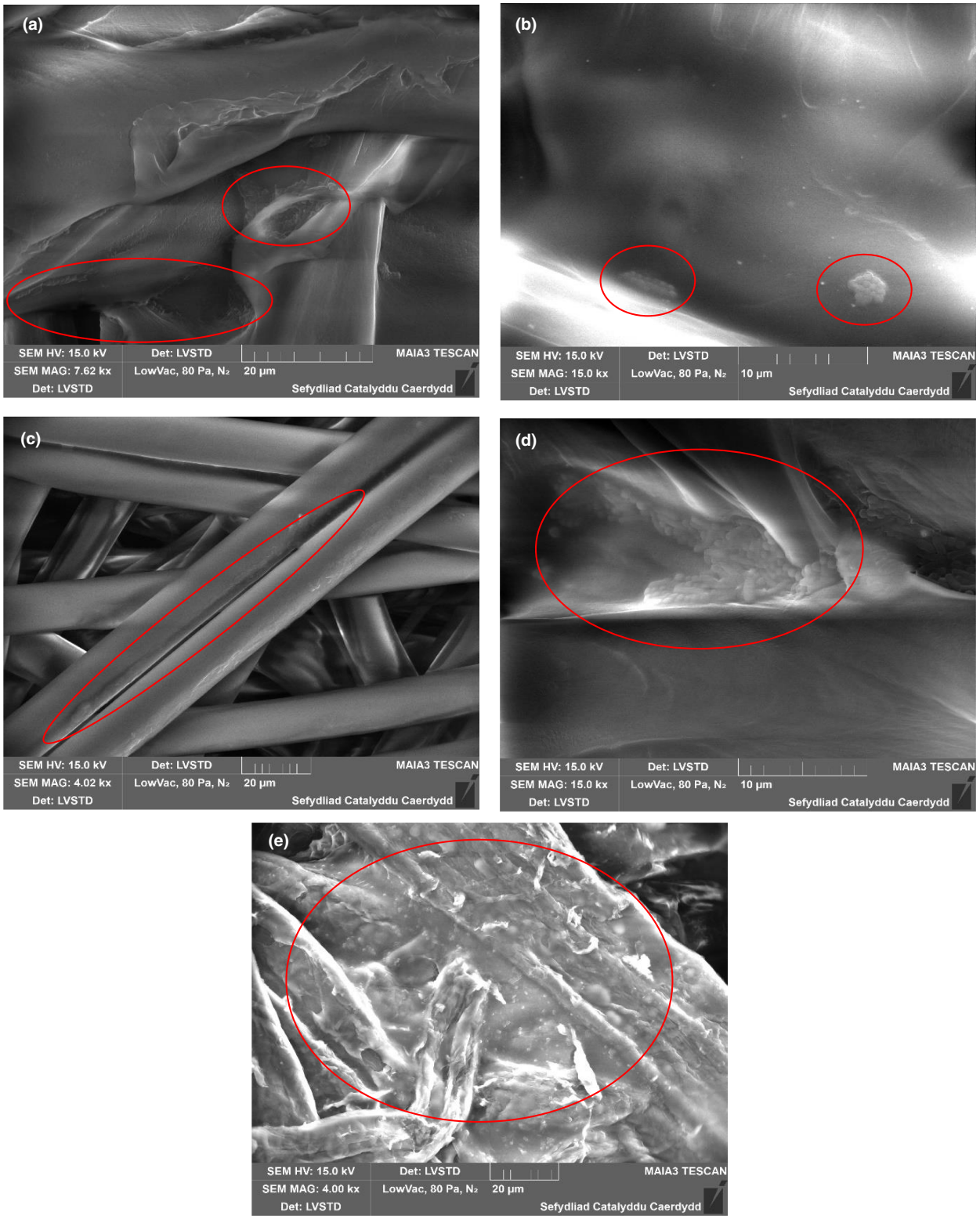

FIGURE 6 Low vacuum scanning electron microscope imaging of wipe materials a–E post-wiping of dual dry surface biofilms (DSB) (*Bacillus licheniformis* and *Staphylococcus aureus*). Red circles indicate presence of bacteria and DSB rafts. (a) Wipe a, (b) wipe B, (c) wipe C, (d) wipe D and (e) wipe E. images taken between ×4000 and ×15,000 magnification

EPS in protecting bacteria from disinfection and drying has been well reported (Almatroudi et al., 2018; Ledwoch et al., 2019; Nkemngong et al., 2020) and is one of the major mechanisms responsible for the decreased biofilm susceptibility to disinfection (Vickery et al., 2012; Xue et al., 2012). Although we did not identify an increased

resistance to disinfection when in a dual species DSB, when considering *B. licheniformis* single species DSB this enhanced resistance to disinfection was observed following all treatments, as we know that *Bacillus* spp. produce higher quantities of EPS to form a robust DSB this resistance could be attributed to the overall structure of DSB.

The efficacy of a surface disinfectant should not only be measured as log reduction in viability, but also as both decreasing bacteria transfer directly or indirectly through cloth/wipe and time taken for biofilm to regrow (Ledwoch et al., 2021). Indeed, accepted efficacy product test standards such as the ASTM 2967–15 and the EN 16615–15 (ASTM E2967, 2015; EN 16615, 2015) measure both reduction in viability and transfer. Our results confirmed the direct transfer of *B. licheniformis* and *S. aureus* from single species DSB, in agreement with previous studies (Ledwoch et al., 2018; Ledwoch et al., 2019; Ledwoch et al., 2021). When dual species were evaluated, a decreased transfer in *S. aureus* was observed (Figure 5). Although there was a reduction in percentage transfer when in a dual species DSB, *B. licheniformis* transfer remained high. Preventing bacterial transfer from a contaminated wipe to another surface overall makes a product safer to use. Again, we observed high variability within the dual dataset for direct surface transfer when considering *S. aureus*; hence, statistics were not performed due to different populations within the dataset. This observation has previously been mentioned when considering dual species DSB culturability.

A high transfer of both species from wipes to a new surface was observed. The effectiveness of microfibre cloths has been previously shown to be not better than that of non-woven conventional cloths, with the added risk of recontaminating surfaces with microorganisms (Moore & Griffith, 2006). Although the removal of bacterial from surfaces using water and cloth was similar to cleaning/disinfectant wipes (Figures 2 and 3), the ability of the water control wipe to transfer bacteria was not. The effectiveness of water on microfibre cloth has previously been investigated by Robertson et al. (2019), who similarly showed that water alone is much less effective at reducing transfer of microorganisms between surfaces and should not be used as a replacement to disinfectant wipes. Our results show that wipe material alone may result in the removal of microorganisms but disinfectants are key to also reducing transfer, rendering the product safer, and the surface safe post-wiping. The spread of bacteria from one surface to another by microfibre cloth has also been described previously and questions the use of this material for environmental cleaning (Bergen et al., 2009). Parvin et al. (2019) investigated the ability of cloth moistened with water to remove *S. aureus* DSB and observed a 1.48 $\log_{10}$ reduction, even with wiping the DSB surface up to 50 times. This reduction is much lower than the results presented here ($\leq$3 $\log_{10}$ reduction with water control), which may be explained with differences in methodology and wipe materials. In our study, DSB are formed by sedimentation in well plates, whilst Parvin et al. (2019) used the CDC Biofilm Reactor. In their study a viscose/polyester blend material was also used to wipe surfaces. It has been reported that, different wipe materials vary greatly in their ability to remove bacteria from surfaces (Boyce, 2021). The presence of dual DSB aggregates on wipe products identified through low vacuum SEM and low transfer rate of bacteria to a new surface suggests that the wipes do not release bacteria to new surfaces assuming correct practice use, one wipe one direction (Williams et al., 2007), is followed.

Although boxplots might not be recommended for use with small data samples (Krzywinski & Altman, 2014), they are used in this study as they are a much better figure for presentation of the data collected to evidence variability of dual DSB.

Here, we have demonstrated that environmental isolates can survive on healthcare surfaces even after cleaning and disinfection protocols. Overall, *S. aureus* was more susceptible to wipe products than *B. licheniformis*. There was no evidence to suggest that *B. licheniformis* protected *S. aureus* from the action of wipe products, indicating that environmental species might not contribute to pathogen protection in a DSB state. When we consider the natural environment, complex DSB found on hospital surfaces have been found to contain up to 18 different bacterial species dominated by Staphylococci and *Bacillus* spp. (Ledwoch et al., 2018). From the results presented here, with two different species of *Bacillus*, the study of dual species DSB may not be sufficient to answer whether a complex multispecies DSB protect pathogens from cleaning and disinfection or not. It is also clear that dual species DSB behave differently from a wet biofilm.

This study outlines environmental species in DSB can easily be transferred from wipe or directly following wiping. In future, it would be of manufacturers' interest to consider testing products against DSB.

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CONFLICT OF INTEREST

No conflict of interest declared.

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