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# Disinfectant tolerance of *Candidozyma auris* and *Candida albicans* biofilms evaluated using the bead assay for biofilms

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**ABSTRACT** *Candidozyma auris* (formerly *Candida auris*) has emerged as a critical nosocomial pathogen, notable for its multidrug resistance and its capability to form biofilms that enable persistence on surfaces. Although effective disinfection strategies are urgently needed, current disinfectant efficacy standards in many regions, such as Europe, are primarily based on testing planktonic *Candida albicans* and do not adequately reflect the resilience of *Candida* biofilms, including those of *C. albicans* and *C. auris*. To address this gap, the Bead Assay for Biofilms, previously developed for bacterial biofilms, was adapted for the first time to eukaryotic cells. The goal was to cultivate *C. auris* and *C. albicans* biofilms and evaluate the efficacy of selected disinfectants across four active substance classes. Cell enumeration demonstrated highly reproducible biofilms, whose architecture was confirmed by scanning electron microscopy. Both an alcohol- and a QAC-based product did not achieve sufficient reduction of at least ≥4 $\log_{10}$ CFU/mL of biofilm-cells when applied under conditions recommended by the manufacturer (alcohol 1 min: *C. auris* 0.82, *C. albicans* 0.54; QAC 1%, 15 min: *C. auris* 1.94, *C. albicans* 0.68). This reduced efficacy is consistent with the known increased tolerance of microorganisms in biofilms. In contrast, peracetic acid and glutaraldehyde achieved sufficient reductions, albeit at relatively high concentrations (peracetic acid 0.1%: *C. auris* 4.75 and 0.05%: *C. albicans* 4.87; glutaraldehyde 0.5%: *C. auris* 5.32 and *C. albicans* 4.15). Our findings underscore the need to adapt disinfection protocols and testing models to consider biofilm formation of *C. auris* and *C. albicans*, and species-specific resilience.

**IMPORTANCE** This study highlights a critical gap in current disinfection efficacy testing standards; many of which rely on planktonic cell models and do not account for the resilience of biofilm-associated cells or emerging pathogens with unique resistance traits. Although species-specific regulatory guidance for *C. auris* exists in certain regions (e.g., in the USA), standardized disinfectant testing remains largely based on suspension assays (often using *C. albicans*) and does not routinely incorporate biofilm models. Using the Bead Assay for Biofilms, we demonstrate that several commonly used disinfectants may fail to inactivate biofilm-associated *C. auris* and *C. albicans* when applied as recommended. This suggests that reliance on planktonic testing may overestimate disinfectant efficacy against clinically relevant pathogenic yeast and highlights the need to expand current testing standards in order to include biofilm-associated pathogens to improve infection prevention strategies. Consequently, our research is of immediate relevance to regulatory bodies, infection control, and public health.

**KEYWORDS** *Candidozyma auris*, *Candida albicans*, biofilm, disinfection, disinfectant efficacy

*Candida* is a genus of yeasts that represents the most prevalent cause of fungal infections worldwide (1). While many *Candida* species are present as part of the normal human microbiota, they have the potential to become opportunistic

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pathogens, especially in immunocompromised individuals. Among them, *Candidozyma auris* (formerly known as *Candida auris* (2)) has emerged as a major global public health concern since it was first identified in 2009 (3). More recently, *C. auris* has been shown to possess multidrug resistance (MDR) properties, and many isolates exhibit resistance to at least one antifungal drug class (4).

*C. auris* has been linked to healthcare-associated outbreaks that are notoriously difficult to control, as cells can persist in hospital settings for months or even years (5, 6). Environmental contamination plays a central role in sustaining these outbreaks, with *C. auris* detected on a wide range of surfaces and reusable medical equipment (5, 7–9). The burden of *C. auris* on patient skin has been directly associated with contamination levels in the surrounding environment (10). Notably, *C. auris* can survive for up to 7 days on moist or dry surfaces and up to 14 days on plastic or porcine skin (11–13), establishing persistent reservoirs for transmission and recontamination.

Within the European Union and the European Economic Area (EU/EEA), the number of *C. auris* cases has nearly doubled between 2020 and 2021: from 335 cases reported in 8 countries in 2020 to 655 cases in 13 countries in 2021, including multiple outbreak events (14–16); however, the actual number of nosocomial transmissions may be even higher (17). Invasive infections caused by *C. auris* have been shown to be associated with high mortality rates of up to 40%, particularly in immunocompromised individuals (18).

In contrast to *C. auris*, *Candida albicans is* generally considered a commensal organism residing in the gastrointestinal and genitourinary tracts (19). However, in immunocompromised individuals, or when the host microbiota is disrupted, *C. albicans* can become pathogenic. It is a leading cause of bloodstream infections, especially in intensive care unit patients and individuals with indwelling devices, such as central venous catheters. Although *C. albicans* generally display lower antifungal resistance compared to *C. auris*, rising resistance to azole-based antifungals is an increasing concern (20, 21).

A key contributing factor to the environmental resilience of *Candida* is its ability to form biofilms (22, 23); structured microbial communities embedded in an extracellular matrix and adherent to biotic and abiotic surfaces (24). In *Candida* biofilms, including those of *C. auris*, the matrix is predominantly composed of mannan-glucan polysaccharides (25–27), which enable the yeast cells to persist for prolonged periods on skin and mucosa, medical devices, and hospital equipment and play a critical role in transmission and infection. Moreover, biofilms protect pathogens against antifungal treatments, host immune responses, and environmental stressors such as desiccation and disinfection (28–32).

Despite the clinical significance of *Candida* biofilms, current standardized methods for evaluating the efficacy of disinfectants remain limited, as they do not take into consideration biofilms and the associated increased difficulties they impose on hygiene management. According to the European standard EN 13624 (33), yeasticidal activity is assessed using a quantitative suspension test in a liquid environment with *C. albicans* as the test organism. While this test provides basic data on disinfection efficacy, it does not replicate conditions present on contaminated surfaces and may not reflect the behavior of emerging pathogens such as *C. auris*. In some regions, including the United States, regulatory guidance has been established for disinfectants effective against *C. auris* (e.g., EPA List P [34]); however, these listings are largely based on suspension-based efficacy data. Surface-based methods such as EN 17387 (35) or EN 16615 (36) provide more realistic testing conditions as they rely on planktonic cells dried onto a surface, but they still do not account for the complex structure and enhanced resilience of *Candida* in biofilms. Although standardized biofilm models do exist, e.g., in the USA (37, 38), they are designed for bacterial biofilms and have not been adapted for use with yeasts. To date, no standardized protocols exist for evaluating the efficacy of disinfectants against *Candida* biofilms, including those formed by *C. albicans* or *C. auris*.

To address this critical gap, the objective of the present study was twofold: First, to adapt the Bead Assay for Biofilms (39, 40) for use as a reproducible and scalable method of cultivating *Candida* biofilms under laboratory conditions, and second, to evaluate the

efficacy of different hospital-grade disinfectants against *C. auris* and *C. albicans* biofilms. Originally developed for bacterial biofilms, the Bead Assay for Biofilms provides a simple and cost-effective alternative to more complex systems such as the CDC Biofilm Reactor (38) or the MBEC Assay (37). It allows reproducible biofilm growth and recovery of viable cells for disinfectant efficacy testing. Disinfectants were selected based on active substance classes commonly used in clinical settings, including alcohol, quaternary ammonium compounds (QAC), peroxide compounds, and aldehydes. Alcohol-based and QAC disinfectant products were tested using concentrations and exposure times that reflect real-world disinfection protocols and manufacturers' recommendations.

## RESULTS

### Repeatability of CFU in untreated biofilms

For the present study, the Bead Assay for Biofilms that was originally developed and validated for bacterial species, such as *Pseudomonas, Salmonella*, and *Klebsiella* (39–41), was adapted for *Candida*. The applicability of the assay was tested on two *Candida* species: *C. auris* DSM 21092 and *C. albicans* DSM 1386. The adapted assay enabled reproducible biofilm formation for both species and yielded consistent colony-forming unit (CFU) counts within biofilms across replicates. Across 14 independent experiments for *C. auris* and 13 independent experiments for *C. albicans*, mean biofilm densities and standard deviations were 6.67 (±0.106) and 6.24 (±0.215) $\log_{10}$ CFU/mL, respectively (Fig. 1). Within individual experiments, means and standard deviations ranged from 6.607 to 6.770 $\log_{10}$ CFU/mL (±0.011 to 0.202) for *C. auris* and from 5.960 to 6.557 $\log_{10}$ CFU/mL (±0.060 to 0.376) for *C. albicans*. ANOVA of untreated control biofilms showed no significant differences between different experiments for either species at a significance level of 0.05.

### Biofilm aggregates on porous glass beads

Scanning electron microscopy (SEM) analysis confirmed the development of biofilm formation on the porous glass beads (PGBs), with yeast cell aggregates attached to the glass surface and residues of the extracellular matrix visible (Fig. 2, matrix indicated with arrows). Aggregates were visible at multiple sites on the glass particles and sometimes were observed to be multi-layered. The morphology of the cells varied, with *C. auris* cells exhibiting an elongated, oval shape with an approximate diameter of 1.5 µm and a length of 3–3.5 µm. In contrast, *C. albicans* cells display a more ovoid morphology with a diameter of 3–4.5 µm. The cell sizes and shapes observed for the *Candida* species tested in the present study are consistent with those reported in previous studies (42–45). The formation of hyphae or para-hyphae was not observed.

### Disinfectant efficacy testing on *Candida* biofilms

This *Candida*-optimized biofilm model was then used to evaluate the efficacy of various disinfectant products against biofilm-embedded cells. As described in detail in the Materials and Methods section, we considered a reduction of ≥4 $\log_{10}$ CFU/mL to be an indicator of successful biofilm disinfection in accordance with international standards used by the medical sector (33, 35, 36). The effective concentration and exposure time required for a successful biofilm disinfection using the Bead Assay for Biofilms are summarized in Table 1. A more detailed overview of distinct cell numbers of controls and treated biofilms and resulting $\log_{10}$ reduction values is given in Table 2.

#### *Disinfection with an alcohol-based product*

The disinfectant efficacy of the alcohol-based product containing ethanol and 1-propanol was tested using exposure times of 1, 5, and 30 min. Within the exposure time recommended by the manufacturer (1 min), neither *C. auris* nor *C. albicans* biofilms demonstrated a successful cell reduction (0.82 and 0.54 $\log_{10}$ CFU/mL, respectively).

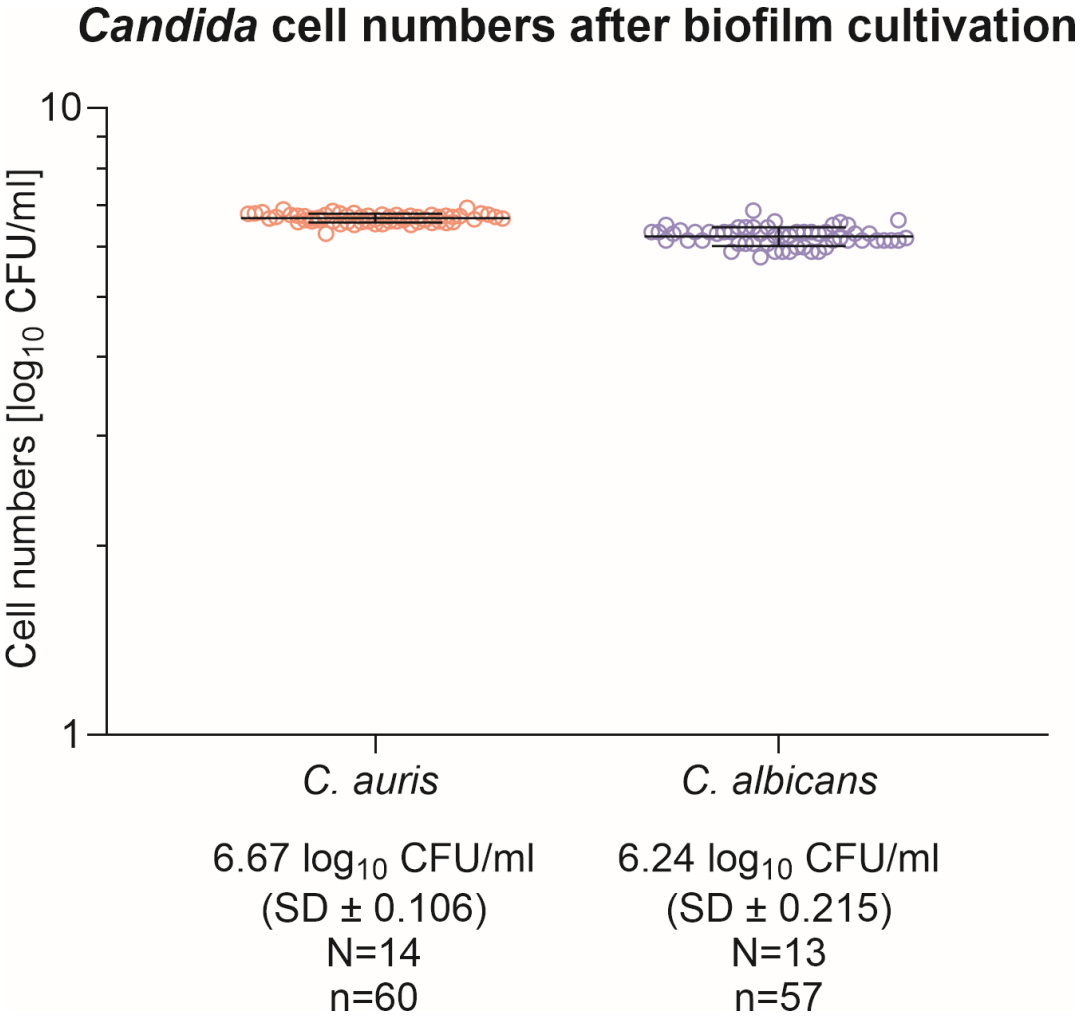


**FIG 1** Cell numbers of untreated biofilms recovered from porous glass beads. Colony-forming units (CFU) are given in $\log_{10}$ CFU/mL of the recovered biofilm suspension. The graph depicts average cell numbers, standard deviation (SD), and individual data points for *C. auris* (red) and *C. albicans* (blue). *N*, number of individual experiments, *n*, number of individual biofilm replicates.



However, after 30 min of exposure, *C. auris* biofilms were reduced by 5.34 $\log_{10}$ CFU/mL, indicating sufficient disinfection (with six replicates out of nine showing complete disinfection). In contrast, *C. albicans* biofilms demonstrated a 1.10 $\log_{10}$ reduction (Table 2; Fig. 3).

### *Disinfection with a quaternary ammonium compound-based product*

In accordance with the manufacturers' recommendations, the efficacy of the QAC-based product was evaluated under two test parameters (1% for 15 min and 0.5% for 30 min). Treatment of *C. auris* biofilms resulted in reductions of 1.94 and 1.37 $\log_{10}$ CFU/mL, respectively, while *C. albicans* biofilms showed reductions of 0.68 and 0.62 log10 CFU/mL (Table 2). Overall, neither conditions achieved the ≥4 $\log_{10}$ CFU/mL reduction required for successful disinfection, indicating that both *C. auris* and *C. albicans* biofilms displayed tolerance toward the QAC-based product (Fig. 4).

### *Disinfection with peracetic acid*

The next substance evaluated was peracetic acid (PAA), an active substance in various yeasticidal disinfectants used for surface disinfection in medical settings. In the present study, PAA was tested at concentrations of 0.01%, 0.05%, 0.1%, and 0.15%, using an

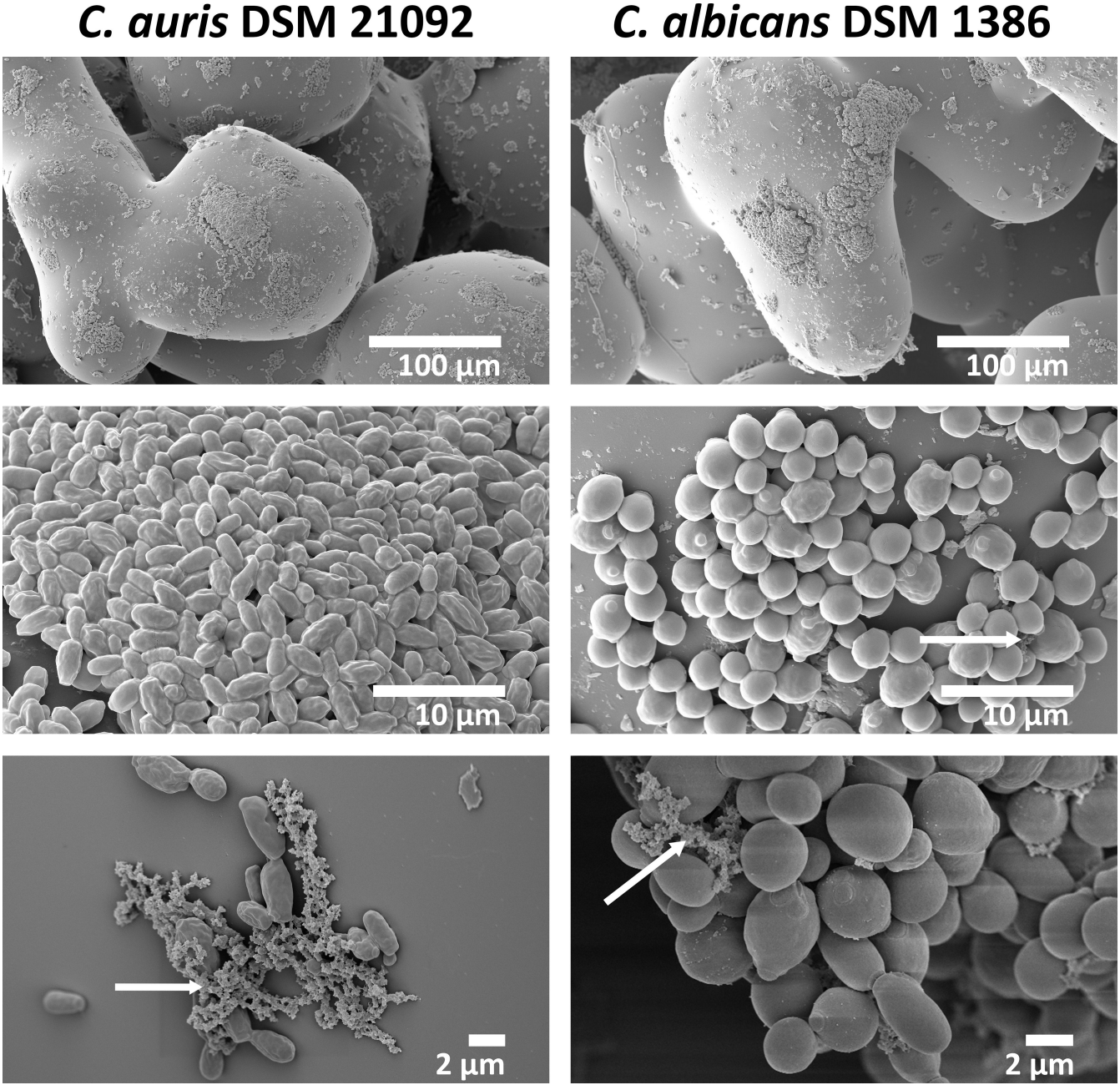


**FIG 2** Scanning electron microscopy images of *C. auris* and *C. albicans* biofilm aggregates grown for 48 h on porous glass beads. Arrows indicate extracellular matrix residues.

exposure time of 10 min. Test parameters were selected based on previous studies that employed the Bead Assay for Biofilms (40, 46, 47). *C. albicans* biofilms were effectively disinfected at 0.05% PAA, achieving a reduction of 4.87 $\log_{10}$ CFU/mL. In contrast, *C. auris* required the next higher concentration tested (0.1%) to reach a comparable reduction (4.75 $\log_{10}$ CFU/mL) (Table 2; Fig. 5). Furthermore, at 0.05% PAA, no growth was observed in 9 out of 12 *C. albicans* replicates, whereas none of the *C. auris* replicates were completely inactivated under the same conditions. These findings highlight potential species-specific differences in biofilm tolerance between *C. auris* and *C. albicans,* the latter being the archetypal test strain for disinfectant efficacy evaluation.

**TABLE 1** Effective concentrations and exposure times for disinfection of *C. auris* and *C. albicans* biofilms obtained with the Bead Assay for Biofilms[a]

| Disinfectant or active substance | Concentration and exposure times evaluated | *Candidozyma auris* | *Candida albicans* |
|---|---|---|---|
| Alcohol-based product | Undiluted[b]; 1, 5, 30 min | 30 min | n.e.[c] |
| QAC-based product | 1% 15 min; 0.5% 30 min | n.e. | n.e. |
| PAA-based product | 0.01, 0.05, 0.1, 0.15%; 10 min | 0.1% | 0.05% |
| Glutaraldehyde | 0.01, 0.05, 0.1, 0.5, 1%; 30 min | 0.5% | 0.5% |

[a]Effective disinfection is assessed when CFU reduction is ≥4 $\log_{10}$ CFU/mL compared to untreated control biofilms.
[b]Ready-to-use product with 23.5% ethanol and 35% 1-propanol.
[c]n.e., not effective; all tested disinfectant concentrations and exposure times were not effective.

### *Disinfection with glutaraldehyde*

The final substance evaluated was glutaraldehyde (GA), an active substance belonging to the group of aldehydes. Similar to PAA, GA is a potent active ingredient found in a range of disinfectant products. However, GA also has a "fixative effect" due to its ability to chemically cross-link proteinaceous structures. Instead of a GA-based product, in the present study, GA was tested as a pure substance and was evaluated at concentrations of 0.01%, 0.05%, 0.1%, 0.5%, and 1%, with an exposure time of 30 min. Both *C. auris* and *C. albicans* biofilms were effectively disinfected with 0.5% GA, with a mean $\log_{10}$ reduction of 5.32 and 4.15 CFU/mL, respectively. However, the cell growth was observed in individual replicates after following treatment despite the high concentrations of GA employed (Table 2; Fig. 6).

## DISCUSSION

In the present study, a reproducible Bead Assay for Biofilms protocol was adapted for cultivation of *Candida* biofilms, and biofilm formation was confirmed quantitatively via CFU counts and qualitatively through scanning electron microscopy. The operational repeatability criterion applied in this study (max. variation of 1 $\log_{10}$ CFU/mL [40]) served as a quality control parameter during assay development to ensure consistent biofilm growth and cell recovery. If variation exceeded the predefined threshold, this was interpreted as an indication that growth conditions or recovery procedures required further optimization or that the assay might not be suitable for the respective test

**TABLE 2** Mean CFU/mL values for non-treated controls and disinfectant-treated samples, expressed as $\log_{10}$, the corresponding mean CFU reduction and the number of technical replicates per condition, where no cell growth had been observed in disinfectant-treated biofilms[a]

| Disinfectant or active substance | | | *Candidozyma auris* | | | | *Candida albicans* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Active substance | Concentration (%) | Time (min) | Mean CFU/mL control | Mean CFU/mL treated | Mean CFU reduction | No growth observed (n/N)[b] | Mean CFU/mL control | Mean CFU/mL treated | Mean CFU reduction | No growth observed (n/N)[b] |
| Alcohol | 23.5 ethanol | 1 | 6.69 | 5.87 | 0.82 | x[c] | 6.23 | 5.70 | 0.54 | x |
| | 35 1-propanol | 5 | 6.58 | 5.02 | 1.56 | x | 6.33 | 5.54 | 0.79 | x |
| | (ready-to-use) | 30 | 6.65 | 1.30 | **5.34** | 6/9 | 6.18 | 5.08 | 1.10 | x |
| QAC | 1 | 15 | 6.69 | 4.74 | 1.94 | x | 6.31 | 5.62 | 0.68 | x |
| | 0.5 | 30 | 6.72 | 5.35 | 1.37 | x | 6.22 | 5.59 | 0.62 | x |
| PAA | 0.01 | 10 | 6.69 | 6.53 | 0.16 | x | 6.20 | 5.86 | 0.34 | x |
| | 0.05 | | | 4.71 | 1.98 | x | | 1.33 | **4.87** | 9/12 |
| | 0.1 | | | 1.94 | **4.75** | 5/15 | | 1.00 | **5.20** | 12/12 |
| | 0.15 | | | 1.24 | **5.44** | 10/12 | | 1.16 | **5.04** | 11/12 |
| GA | 0.01 | 30 | 6.69 | 6.57 | 0.12 | x | 6.29 | 6.37 | −0.07 | x |
| | 0.05 | | | 5.63 | 1.06 | x | | 6.15 | 0.14 | x |
| | 0.1 | | | 4.69 | 2.00 | x | | 5.60 | 0.69 | x |
| | 0.5 | | | 1.36 | **5.32** | 7/9 | | 2.14 | **4.15** | 4/9 |
| | 1 | | | 1.30 | **5.39** | 7/9 | | 1.94 | **4.36** | 3/9 |

[a]Bold—effective disinfection achieved (mean CFU reduction ≥4 $\log_{10}$).
[b]Number of technical replicates (from a total of 9/12/15) with no viable cells observed after disinfectant treatment ($\log_{10}$ = 1).
[c]x = cell growth observed on all disinfectant-treated replicates.

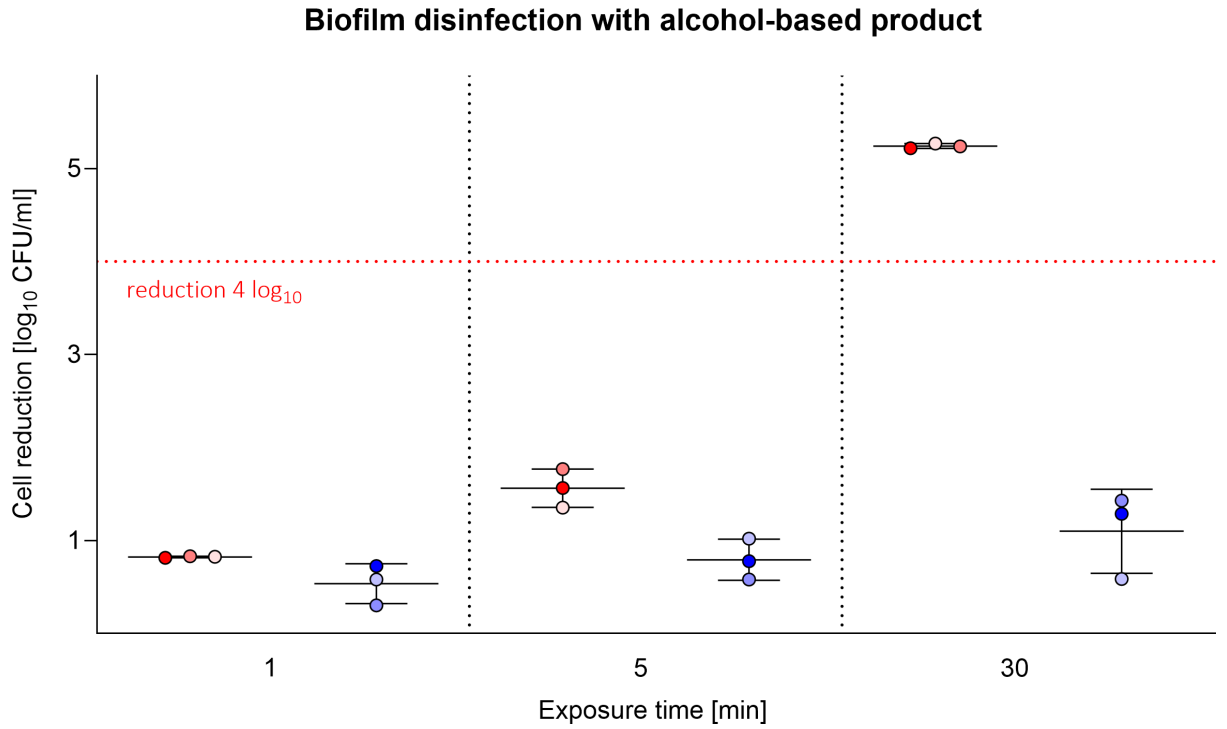


**FIG 3** Efficacy of an alcohol-based disinfectant (ethanol and 1-propanol) against *C. auris* (red) or *C. albicans* (blue) biofilms, evaluated using the Bead Assay for Biofilms. The graph shows the mean $\log_{10}$ reduction in CFU/mL of recovered biofilm cells, including standard deviation, following 1, 5, or 30 min of exposure at 20 °C compared to untreated controls. Individual data points represent the mean of three individual experiments with three technical replicates each. The dashed line indicates a 4 $\log_{10}$ reduction in CFU.

organisms. Importantly, this criterion was not applied to disinfectant-treated samples. Subsequent ANOVA-based analysis also confirmed repeatability-assertions defined in other publications (39, 48). This reliable and highly reproducible biofilm cultivation assay permitted the evaluation of disinfectant efficacy against *Candida* biofilms, thereby enabling the quantification of viable cells post-treatment. Findings from a previous study align well with the results of our study, demonstrating the general applicability of porous glass beads for the cultivation of *Candida* spp. biofilms (45).

The present study revealed reduced disinfectant efficacy against biofilm-associated cells. The efficacy claims for the tested products are based on standard planktonic models, namely, EN 13624 and EN 16615, employing *C. albicans* DSM 1386 as the test organism (33, 36). Importantly, none of the products evaluated here claims specific anti-biofilm activity, and the reduced efficacy observed is consistent with the well-known increased tolerance of microorganisms in biofilms. However, this distinction may not always be apparent to end-users interpreting disinfectant efficacy claims. In line with this, in our model, both the QAC-based product and the alcohol-based product failed to achieve effective disinfection of *C. auris* and *C. albicans* biofilms when applied at manufacturer-recommended conditions. For instance, *C. auris* biofilms could only be effectively disinfected with the alcohol-based product after 30 min of exposure, a duration impractical for routine surface disinfection due to rapid alcohol evaporation. These findings are consistent with previous studies reporting reduced susceptibility of *C. auris* to certain disinfectants, particularly QAC-based products (49–52). In contrast, PAA and GA achieved effective disinfection, albeit at relatively high concentrations comparable to those necessary for disinfection of *P. aeruginosa*, *K. pneumoniae,* or *S. enterica* biofilms (39, 40, 47). This suggests that additional consideration is necessary when selecting disinfectants in settings where biofilm formation is likely.

One of the main findings of this study was the observation of species-specific differences in biofilm tolerance between *C. albicans* and *C. auris*, particularly during PAA

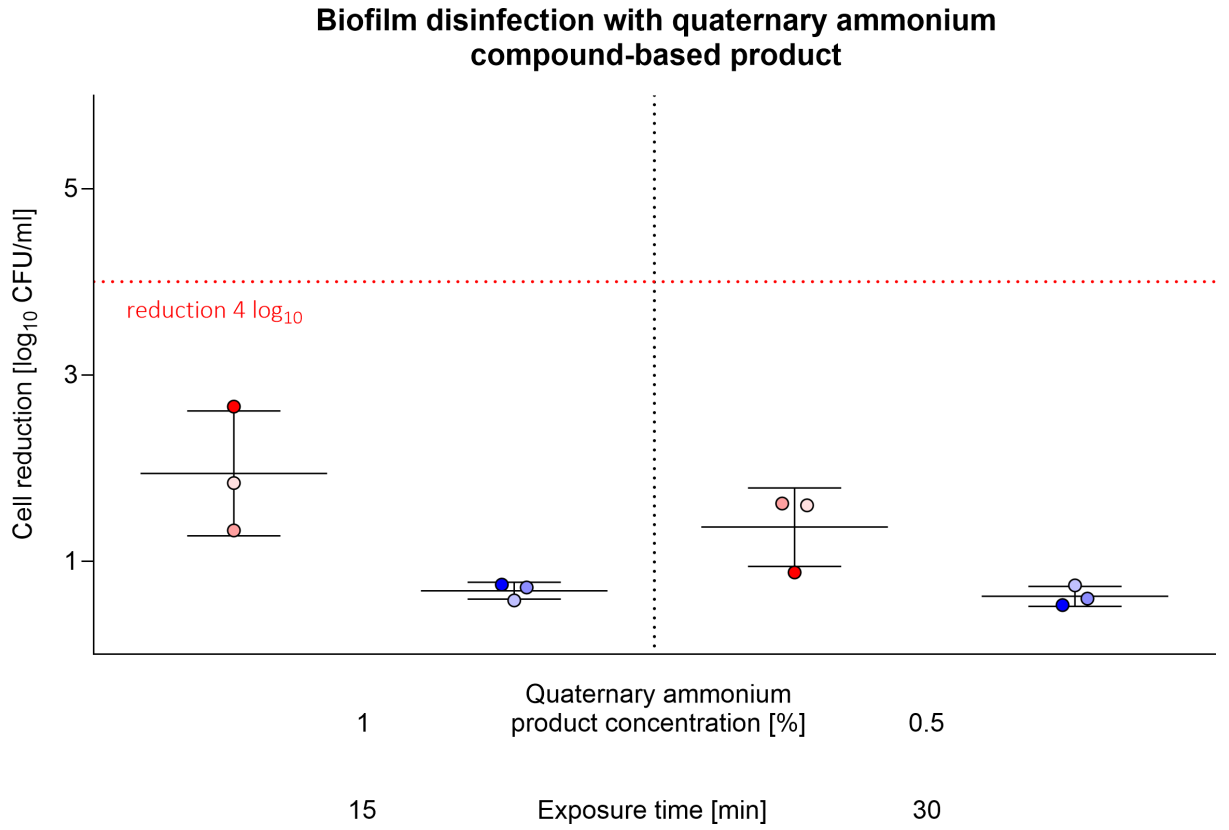


**FIG 4** Efficacy of a quaternary ammonium compound (QAC)-based disinfectant against *C. auris* (red) or *C. albicans* (blue) biofilms evaluated with the Bead Assay for Biofilms. The graph shows the mean $\log_{10}$ reduction in CFU/mL of recovered biofilm cells, including standard deviation, after treatment under two conditions (1% for 15 min or 0.5% for 30 min, 20°C) compared to untreated controls. Individual data points represent the mean of three individual experiments with three technical replicates each. The dashed line indicates a 4 $\log_{10}$ reduction in CFU.

disinfection. Due to its high antimicrobial efficiency and decomposition into non-toxic byproducts, PAA is widely used in healthcare settings, including surface disinfection and medical device reprocessing. In the present study, higher concentrations of PAA were required for disinfection of *C. auris* biofilms compared to *C. albicans*. These observations may reflect intrinsic biological variation in biofilm architecture, extracellular matrix composition, and stress response between the two species. Similar variability has been reported at the strain level in other organisms, such as *K. pneumoniae* and *S. enterica* biofilm models (41, 46, 47, 53). This observation is particularly important since *C. albicans* is the standard test strain used for evaluating the effectiveness of yeasticidal products, particularly in European Standardization. Therefore, the present study suggests that reliance on *C. albicans* as the sole yeast test strain may, therefore, not fully capture species-specific differences in disinfectant tolerance. This is especially important considering that *C. auris* currently poses a greater threat for nosocomial yeast infections and outbreaks than other members of the Candida genus. Furthermore, the present study highlights a need for standardized but flexible and adaptable testing approaches that account for biofilm-associated growth and interspecies variability when evaluating disinfectant efficacy.

Our findings indicate that increasing disinfectant concentrations or exposure times is not a universally applicable solution for addressing Candida biofilms, particularly in real-world healthcare settings with strict time constraints and reliance on volatile compounds, such as alcohol. Instead, a multi-faceted approach is required to prevent and combat biofilm formation. This may include adapting disinfection protocols to incorporate mechanical measures (e.g., flushing, scrubbing, or ensuring adequate surface

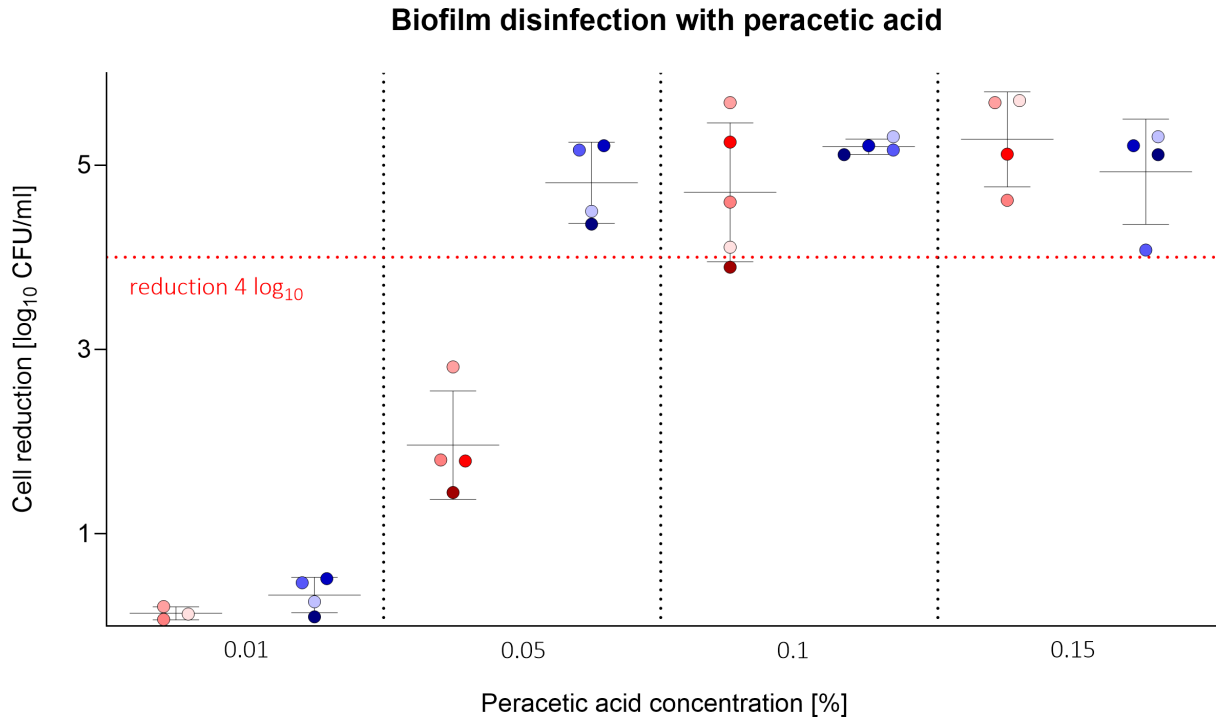


**FIG 5** Disinfection efficacy of peracetic acid (PAA) against *C. auris* (red) or *C. albicans* (blue) biofilms evaluated with the Bead Assay for Biofilms. The graph shows the mean $\log_{10}$ reduction in CFU/mL of recovered biofilm cells, including standard deviation, after treatment with 0.01%, 0.05%, 0.1%, or 0.15% PAA for 10 min at 20°C compared to untreated controls. Individual data points represent the mean of three individual experiments with three technical replicates each. The dashed line indicates a 4 $\log_{10}$ reduction in CFU.

drying) alongside optimized chemical strategies. In practice, the transition to more potent products or expanding the spectrum of substance classes may be more effective than simply increasing the dose of conventional agents, particularly when hygiene protocols are intensified due to a special indication. Based on our findings, alternative substances such as peracetic acid or glutaraldehyde may be considered where alcohol- or QAC-based disinfectants prove ineffective: for example, during outbreak situations or where biofilm formation is likely.

The present study has several limitations. The study focused exclusively on two Candida species, with each species represented by a single strain. Moreover, the *C. auris* isolate used in this study (DSM 21092) belongs to Clade II, which has been reported to exhibit higher susceptibility to certain disinfectants than isolates from other clades (49, 50). Consequently, the tolerance observed here may represent a conservative estimate of the challenge associated with disinfecting *C. auris* biofilms. Nevertheless, the increased tolerance of biofilm-associated microorganisms compared with planktonic cells is well established, suggesting that *Candida* biofilms may generally be more difficult to inactivate than cells evaluated in conventional suspension- or surface-based disinfectant efficacy tests. In this study, disinfectant efficacy was quantified using CFU enumeration to maintain comparability with established disinfectant testing standards. European standards (e.g., EN 13624 and EN 16615) and U.S. biofilm-specific methods such as, e.g., ASTM E2799 and ASTM E2562 (37, 38), also rely on CFU reduction as the primary endpoint for assessing antimicrobial efficacy. Because disinfection aims to achieve microbial killing rather than merely biomass reduction, viability-based quantification was considered the most appropriate measure for evaluating disinfectant efficacy. In addition, comparative analysis using non-biofilm standards (e.g., EN 16615 or EN 13624) might provide additional clarity regarding the transferability of results obtained with *C. albicans* to *C. auris*. Finally, the present study evaluated disinfectants belonging to four distinct active substance classes; however, only one product per substance class was

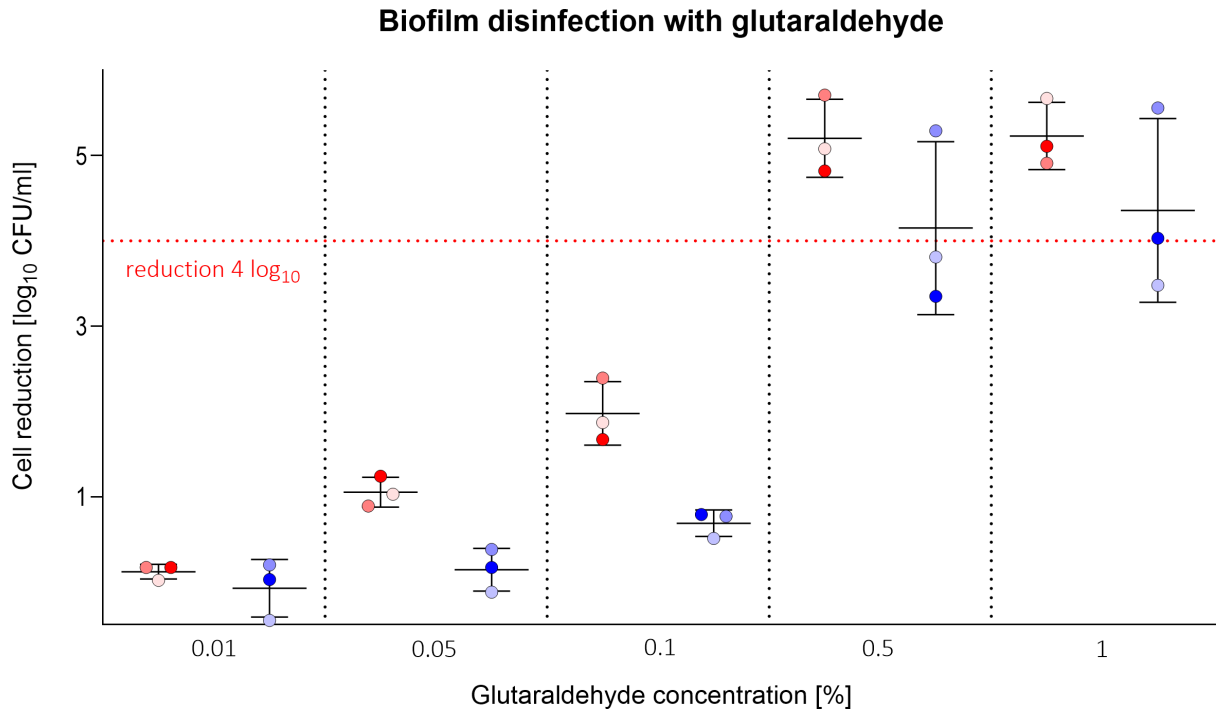


**FIG 6** Disinfection efficacy of glutaraldehyde (GA) against *C. auris* (red) or *C. albicans* (blue) biofilms evaluated with the Bead Assay for Biofilms. The graph shows the mean $\log_{10}$ reduction in CFU/mL of recovered biofilm cells, including standard deviation, after treatment with 0.01%, 0.05%, 0.1%, 0.5%, or 1% glutaraldehyde for 30 min at 20°C compared to untreated controls. Individual data points represent the mean of three individual experiments with three technical replicates each. The dashed line indicates a 4 $\log_{10}$ reduction in CFU.

tested and, thus, may not be representative for other products from the same substance class. Therefore, future studies should test a broader range of disinfectant formulations and place particular emphasis on species-specific differences of *C. auris* and *C. albicans*.

In summary, the present study demonstrates that disinfectant efficacy based on planktonic *C. albicans* may not fully reflect the tolerance of cells residing in biofilms. These findings highlight the importance of considering biofilm-associated microorganisms when evaluating disinfectant performance. In particular, the observed differences in disinfectant tolerances between *C. auris* and *C. albicans* suggest that reliance on a single yeast reference strain in standardized testing may not always capture the variability of clinically relevant pathogens. Expanding testing approaches to include biofilm models may, therefore, contribute to a more realistic assessment of disinfectant efficacy in healthcare environments. Ultimately, effective infection prevention strategies should address both cleaning and disinfection to impede the formation of resilient biofilms, particularly those formed by multidrug-resistant pathogens such as *C. auris*.

## MATERIALS AND METHODS

### Yeast strains

Biofilm-associated yeasts were grown using *Candida auris* DSM 21092 and *Candida albicans* DSM 1386 (ATCC 10231) reference strains. Yeast cells were stored at −80°C, in glycerol stock solutions at a concentration of $1 \times 10^9$ colony-forming units (CFU)/mL. Prior to each experiment, frozen stocks were thawed and diluted 1:50 in Malt Extract Broth (MEB), followed by overnight incubation at 37°C with shaking at 250 rpm.

## Biofilm cultivation and disinfectant efficacy testing

Biofilm cultivation and subsequent disinfection efficacy testing were conducted using the Bead Assay for Biofilms as previously described (39, 40). Briefly, biofilms were cultivated on 4 mm porous glass beads (PGB) (Sinterglas Pellets, ROBU Glasfilter-Geräte GmbH, Hattert, Germany) in 24-well microplates (one bead per well). An overnight culture of *C. auris* or *C. albicans* was diluted in MEB to approximately $1 \times 10^5$ CFU/mL and dispensed into the bead-containing 24-well microplate (1 mL per well). The microplate was then incubated at 30°C for 48 h at 100 rpm in an orbital shaker. After biofilm cultivation, each bead was then carefully dipped into 2 mL sterile $H_2O$ to remove non-adherent cells and placed in a 2 mL microcentrifuge tube containing 0.2 mL of disinfectant. Controls were treated with hard water (HW), which was prepared according to EN 13624 (33), instead of disinfectant. After the defined exposure time, 1.8 mL of neutralizing agent was added. Subsequently, yeast cells were treated by sonication in an ultrasonic bath (BactoSonic, Bandelin, Berlin, Germany) at 40 kHz for 10 min using 200 $W_{eff}$ to detach biofilm cells from the bead surface. Recovered yeast cells were quantified by serial dilution in neutralizing agent. Here, 500 µL of each sample was transferred to the "0" row wells of a 96-well microplate (Eppendorf deepwell Plates 96/2,000 µL). All samples were then diluted from row "0" through row "−6" in 1:10 steps (150 µL sample + 1.350 mL neutralizing solution) using a multichannel pipette. Subsequently, 5 µL of every dilution was spot deposited onto a square Malt Extract Agar (MEA) plate (12 × 12 cm) using a multichannel pipette. Plates were incubated overnight at 30°C and CFU/mL were counted after 48 h. Only spotting areas with 5 to 50 CFUs were counted. Independent experiments were performed on different days, each initiated from a fresh glycerol stock to ensure biological independence. Each condition (e.g., control, disinfectant concentration) was tested at least three times with three technical replicates each. During assay development, repeatability (intra-laboratory precision) was assessed using untreated control biofilms and was operationally defined as a maximal variation of ≤1 $\log_{10}$ CFU/mL between independent experiments (40). In addition, repeatability was supported by one-way analysis of variances (ANOVA) of control biofilms, allowing the assessment of variability between experiments and confirming consistency of biofilm growth and cell recovery (39, 48).

## Disinfection and neutralization conditions

For disinfection experiments, biofilms were exposed to (i) an alcohol-based product (23.5% ethanol and 35% 1-propanol) for an exposure time of 1-, 5- or 30-min; (ii) a quaternary ammonium compound (QAC) product (7.5% benzalkonium chloride, 10% 2-phenoxyethanol, 8% N,N-bis-(3-aminopropyl)dodecylamine) at a concentration of 0.5% for 30 min or a concentration of 1% for 15 min; (iii) peracetic acid (PAA, Lerasept Spezial, Stockmeier Chemie, Bielefeld, Germany) at concentrations of 0.2%, 0.15%, 0.1%, 0.05%, and 0.01% PAA for 10 min; and (iv) glutaraldehyde (GA, Glutaraldehyde 50%, Sigma Aldrich, Saint Louise, MI, USA) at a concentration of 1%, 0.5%, 0.1%, 0.05%, and 0.01% GA for 30 min. After the indicated exposure times, disinfectant solutions were neutralized for 5 min in the following solutions: the alcohol-based product was neutralized with PBS (0.1 M, pH 7); PAA with a solution of 1.65% sodium sulfite (Sigma Aldrich, Saint Louise, MI, USA) in PBS (0.1 M, pH 7); GA and QAC-based products with 20 g/L glycine and 10 g/L Tween 80 in 0.25 M PBS. Disinfection and neutralization were performed at 20 ± 1°C. Disinfectants were stored according to the manufacturer recommendations and, prior to analysis, were diluted with HW. According to the EN 13624 standard used here, the final water hardness depends on the particular test product utilized; however, it is less than 375 mg/L of calcium carbonate ($CaCO_3$) (33). For the validation of neutralization and to verify the absence of toxicity, respective neutralizers were tested against the highest product test concentration used (for QAC 1%, PAA 0.2%, and for GA 1%, all wt/wt, concentrated alcohol-based product), and for both test strains according to EN 13624 (33).

## Evaluation of disinfectant efficacy

In the present study, a ≥4 $\log_{10}$ reduction in CFU is defined as the threshold for successful disinfection, which is in accordance with current standards evaluating yeasticidal products in the medical sector (54). CFU values represent viable cells recovered from biofilms after sonication and are expressed as CFU/mL. Disinfectant efficacy is represented by the log reduction (R) in CFU, which is calculated according to the following equation: $\log_{10}R = \log_{10} N_0 - \log_{10} N$ ($\log_{10} N_0$ = logarithm of the CFU for the untreated control; $\log_{10} N$ = logarithm of the CFU after disinfection). In order to be able to detect a 4 $\log_{10}$ reduction in yeast count after treatment with disinfectant, an initial CFU per mL of $\geq 10^5$ was defined as a requirement for disinfectant testing. The assay-specific limit of detection was set to 1 $\log_{10}$ (or 10 CFU/mL) and was applied when no viable cells were observed. This value depends on the initial volume in which the biofilm was resuspended, the serial dilution, and the final volume that was spread onto the agar plates to determine the CFU. It was, therefore, calculated from the logarithm of the dilution factor of the biofilm cells used for quantification.

## Scanning electron microscopy imaging

For Scanning Electron Microscopy (SEM) imaging of *Candida* spp. biofilms on porous glass beads, biofilm cultivation was performed as described above. After 48 h, biofilms were fixed in a solution of 1% paraformaldehyde and 2.5% glutaraldehyde in 50 mM HEPES buffer for 4 days at room temperature. Subsequently, all samples were dehydrated in 30, 50, 70, 90, 95, and twice 100% ethanol, dried in hexamethyldisilane overnight, mounted on aluminum stubs with Acheson Silver (DAG 1415M), sputter coated with a 10 nm layer of gold-palladium, and finally examined by SEM (Teneo VS, FEI Germany GmbH) operated at 10 kV using an ET detector.

## Data analysis and visualization

Data analysis and visualization were performed using PRISM 9.0 GraphPad Software (Version 9.1.0, La Jolla, CA, USA).

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