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Padronização de ensaios de adesão e eficácia antibiofilme de antifúngicos e clorexidina frente
a organismos relacionados à ceratite infecciosa grave

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Trabalho de Conclusão de Curso de Graduação em Farmácia do Centro de Ciências da Saúde, Departamento de Análises Clínicas, da Universidade Federal de Santa Catarina como requisito para a obtenção do título de Bacharel em Farmácia.

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Este Trabalho de Conclusão de Curso foi julgado adequado para obtenção do título de Farmacêutico e aprovado em sua forma final pelo Curso de Farmácia da UFSC.

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“A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original.”

— Albert Einstein

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RESUMO

Ceratites infecciosas graves estão entre as principais causas de transplantes de córnea e perda de visão, ocasionadas por microrganismos como o protozoário do gênero *Acanthamoeba* e leveduras como *Candida* spp. O tratamento dessas infecções é desafiador, especialmente nos casos de ceratite polimicrobiana, nos quais múltiplos microrganismos, incluindo protozoários e fungos, estão envolvidos simultaneamente. Tanto *Acanthamoeba* spp. quanto as espécies de *Candida* podem aderir às lentes de contato e à córnea do paciente, levando à formação de biofilmes polimicrobianos. No entanto, estudos sobre o potencial de adesão de *Acanthamoeba* spp. são escassos. Assim, o objetivo deste estudo foi padronizar metodologias para avaliar a adesão e formação de biofilmes entre *Acanthamoeba* spp. e *Candida albicans*, além de analisar a eficácia antibiofilme da natamicina, do voriconazol e da clorexidina contra esses microrganismos. A capacidade de adesão foi avaliada utilizando o corante cristal violeta, enquanto a suscetibilidade aos fármacos foi determinada com o uso do corante Tripan Blue. Os resultados mostraram que tanto a cepa de *A. castellanii* quanto o isolado clínico R40 apresentaram alta capacidade de adesão. Todos os tratamentos testados demonstraram atividade antibiofilme promissora e foram eficazes na erradicação de biofilmes de *C. albicans* e *Acanthamoeba* spp. A natamicina se destacou na redução de biofilmes de *C. albicans*, tanto isoladamente quanto em co-cultura com *Acanthamoeba* spp., enquanto a clorexidina mostrou-se altamente eficaz na erradicação de biofilme de *Acanthamoeba* spp. Portanto, um melhor entendimento do comportamento de adesão de *Acanthamoeba* spp., isoladamente ou em associação com espécies de *Candida*, e a avaliação da eficácia antibiofilme dos tratamentos podem contribuir para um manejo mais eficaz da ceratite polimicrobiana grave, como também a avaliação da eficácia de soluções multipropósitos para a higienização e manutenção das lentes de contato e estojos.

Palavras-chave: ceratite polimicrobiana; *Acanthamoeba*; *Candida*; biofilme microbiano; atividade antibiofilme.

ABSTRACT

Severe infectious keratitis is among the leading causes of corneal transplants and vision loss, caused by microorganisms such as the protozoan genus *Acanthamoeba* spp. and yeasts *Candida* spp. Treating these infections is challenging, especially in cases of polymicrobial keratitis, in which multiple microorganisms, including protozoa and fungi, are involved simultaneously. Both *Acanthamoeba* spp. and *Candida* species can adhere to contact lenses and the patients cornea, leading to the formation of polymicrobial biofilms. However, studies on the adhesion potential of *Acanthamoeba* spp. are scarce. Therefore, this study aimed to standardize methodologies to evaluate adhesion and biofilm formation between *Acanthamoeba* spp. and *Candida albicans*, as well as to analyze the antibiofilm efficacy of natamycin, voriconazole, and chlorhexidine against these microorganisms. Adhesion capacity was assessed using crystal violet dye, while drug susceptibility was determined using Trypan Blue dye. The results showed that the strain *A. castellanii* and the clinical isolate R40 exhibited high adhesion capacity. All treatments tested demonstrated promising antibiofilm activity and were effective in eradicating biofilms of *C. albicans* and *Acanthamoeba* spp. Natamycin stood out in reducing biofilms of *C. albicans*, both alone and in co-culture with *Acanthamoeba* spp., while chlorhexidine proved highly effective in eradicating *Acanthamoeba* spp. biofilms. Therefore, a better understanding of the adhesion behavior of *Acanthamoeba* spp., either alone or in association with *Candida* species, and the evaluation of the antibiofilm efficacy of treatments could contribute to more effective management of severe polymicrobial keratitis, as well as the assessment of the efficacy of multipurpose solutions for cleaning and maintaining contact lenses and cases.

Keywords: polymicrobial keratitis; *Acanthamoeba*; *Candida*; microbial biofilm; antibiofilm activity.

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Standardization of adhesion and antibiofilm efficacy assays of antifungals and chlorhexidine against
microorganisms associated with severe infectious keratitis

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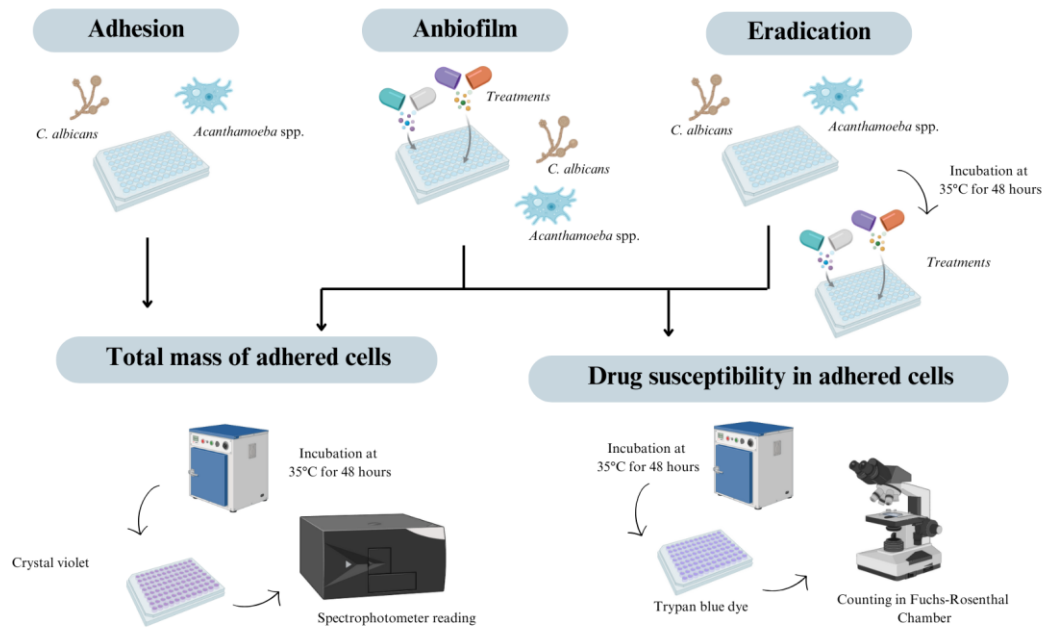
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ABSTRACT

Severe infectious keratitis is among the leading causes of corneal transplants and vision loss, caused by microorganisms such as the protozoan genus *Acanthamoeba* spp. and yeasts *Candida* spp. Treating these infections is challenging, especially in cases of polymicrobial keratitis, in which multiple microorganisms, including protozoa and fungi, are involved simultaneously. Both *Acanthamoeba* spp. and *Candida* species can adhere to contact lenses and the patients cornea, leading to the formation of polymicrobial biofilms. However, studies on the adhesion potential of *Acanthamoeba* spp. are scarce. Therefore, this study aimed to standardize methodologies to evaluate adhesion and biofilm formation between *Acanthamoeba* spp. and *Candida albicans*, as well as to analyze the antibiofilm efficacy of natamycin, voriconazole, and chlorhexidine against these microorganisms. Adhesion capacity was assessed using crystal violet dye, while drug susceptibility was determined using Trypan Blue dye. The results showed that the strain *A. castellanii* and the clinical isolate R40 exhibited high adhesion capacity. All treatments tested demonstrated promising antibiofilm activity and were effective in eradicating biofilms of *C. albicans* and *Acanthamoeba* spp. Natamycin stood out in reducing biofilms of *C. albicans*, both alone and in co-culture with *Acanthamoeba* spp., while chlorhexidine proved highly effective in eradicating *Acanthamoeba* spp. biofilms. Therefore, a better understanding of the adhesion behavior of *Acanthamoeba* spp., either alone or in association with *Candida* species, and the evaluation of the antibiofilm efficacy of treatments could contribute to more effective management of severe polymicrobial keratitis, as well as the assessment of the efficacy of multipurpose solutions for cleaning and maintaining contact lenses and cases.

Keywords: polymicrobial keratitis; *Acanthamoeba*; *Candida*; microbial biofilm; antibiofilm activity.

GRAPHICAL ABSTRACT



1. INTRODUCTION

In the global context of eye health, keratitis infections are a leading cause of blindness in humans (Ung *et al.*, 2019). This condition presents a major public health challenge since it can significantly affect patients vision and overall quality of life (Ting *et al.*, 2021). *Acanthamoeba* keratitis (AK) is an eye infection caused by *Acanthamoeba* species. Polymicrobial keratitis (PK), on the other hand, can occur when multiple microorganisms, including fungi and bacteria, are present alongside the amoeba. Both conditions are known for their clinical complexity and resistance to standard drugs (Jones, 1981; Ahn *et al.*, 2011; Lorenzo-Morales; Khan; Walochnik, 2015).

Both AK and PK are commonly linked to predisposing factors, such as contact lenses use, eye trauma, compromised ocular surface, and immunosuppression, which may be present in HIV-positive individuals or those who have undergone transplants (Buchele *et al.*, 2018; Khor *et al.*, 2018; Rayamajhee *et al.*, 2021; Ung *et al.*, 2019). The incidence of these infections varies significantly around the world. In the United States of America, the rate is 11 cases per 100,000 inhabitants. In contrast, developing countries experience much higher rates. For instance, in Nepal the incidence reaches an alarming 799 cases per 100,000 inhabitants each year (Sakr *et al.*, 2023). Current ophthalmic formulations for treating AK are ineffective, leading to recurrent infections, treatment failures, and prolonged therapy (Maycock; Jayaswal, 2016). The most commonly used drugs include polyhexamethylene biguanide (PHMB), chlorhexidine gluconate, diamidines, propamidine isethionate (Brolene®), and hexamidine (Desomedine®) (Lim *et al.*, 2008; Dart; Saw; Kilvington, 2009; Elsheikha; Siddiqui; Khan, 2020).

Voriconazole and natamycin are commonly used to treat keratomycosis, especially those caused by *Candida* spp. (Prajna *et al.*, 2010; Sharma *et al.*, 2013). Although natamycin eye drops is approved by the Food and Drug Administration (FDA), it has not yet received approval from the National Agency for Sanitary Monitoring (ANVISA). It is currently only available in compounded formulations (Prajna *et al.*, 2010). Developing fungal biofilms contributes to pathogenicity and enhances resistance to commercial antifungal treatments (Ramage *et al.*, 2009; Zhang *et al.*, 2012; Córdova-Alcántara *et al.*, 2019). These structures are complex communities and exhibit a higher level of organization than individual (planktonic) cells, offering significant advantages to the organisms that inhabit them (Flemming; Wuertz, 2019).

For *Acanthamoeba* spp., biofilms on contact lenses and at infection sites provide protection, dissemination, and resistance (Lorenzo-Morales; Khan; Walochnik, 2015). Some studies indicate that biofilms resist multipurpose cleaning and disinfecting solutions for contact lenses, which promotes the adhesion of

Acanthamoeba spp. to lens surfaces (Imamura *et al.*, 2008; Lorenzo-Morales; Khan; Walochnik, 2015; Pinto *et al.*, 2021).

Thus, this is the first study in the literature to explore polymicrobial biofilm formation involving *Acanthamoeba* and *Candida*. It aims to standardize methodologies for evaluating the potential adhesion and biofilm formation between *Acanthamoeba* spp. and *Candida albicans*, as well as to assess the effectiveness of antifungal agents and chlorhexidine against these microorganisms.

2. MATERIALS AND METHODS

2.1 Antifungals and chlorhexidine

To evaluate antibiofilm activity and biofilm eradication, we used two antifungal agents: voriconazole (Acros Organics, Thermo Fisher Scientific, New Jersey, USA) and natamycin (5% Eye Drops - Oftálmica Manipulação, Porto Alegre, RS, Brazil), as well as chlorhexidine 0.2% (Riohex®). The stock solution of voriconazole was prepared in dimethyl sulfoxide (DMSO, Neon®) and subsequently serially diluted in RPMI 1640 medium (Gibco®) to achieve a final concentration of 32 µg/mL, ensuring that the DMSO concentration did not exceed 2%. The natamycin solution was diluted directly in RPMI 1640 medium from the drug bottle to reach the same final 32 µg/mL. For chlorhexidine, a 200 µM solution was prepared in both RPMI 1640 medium, and PYG medium [0.75% (wt/vol) proteose peptone, 0.75% (wt/vol) yeast extract, and 1.5% (wt/vol) glucose] directly from the drug bottle.

2.2 Protozoa culture and collection

In the anti-adhesion and biofilm eradication assays of antifungals and chlorhexidine, a regional clinical isolate of *Acanthamoeba* spp. (R40 - genotype T4) was obtained from corneal scrapings of a patient diagnosed with *Acanthamoeba* keratitis and characterized at the Laboratory of Applied Research in Emerging Protozoa at the Federal University of Santa Catarina (Certificate of Presentation for Ethical Appreciation - CAAE 32938014.5.0000.0121). Additionally, *A. castellanii* ATCC 50492 strain (genotype T4) was used. The standard strain and the *Acanthamoeba* spp. isolate was cultured axenically in PYG medium at 30°C for 5 days (Schuster, 2002).

2.3 Fungal strain culture and collection

Candida albicans strain (Ca MT01) was utilized to assess the antibiofilm activity and biofilm eradication capabilities of the antifungals voriconazole (Acros Organics, Thermo Fisher Scientific, New Jersey, USA) and natamycin (5% Eye Drops - Oftálmica Manipulação, Porto Alegre, RS, Brazil). This strain is part of the organism collection at the Laboratory of Applied Mycology Research at the Federal University of Rio Grande do Sul. Before the experiment, *C. albicans* was cultured on Sabouraud Agar containing Chloramphenicol at 35°C for 24 hours.

2.4 Evaluation of *Acanthamoeba castellanii* strain, the clinical isolate, and *Candida albicans* adhesion, alone and in co-culture.

This assay evaluated the adhesion capacity of *Acanthamoeba castellanii* (ATCC 50492) strain, R40 isolate of *Acanthamoeba* spp., and *C. albicans*, both individually and in co-culture. After the culture of each strain, the trophozoites and yeast cell counts were conducted using a Fuchs-Rosenthal chamber to prepare an inoculum suspension with approximately 8×10^5 cells/mL and 8×10^5 CFU/mL for *Acanthamoeba* spp. and *C. albicans*, respectively, both using PYG and RPMI media. For individual *Acanthamoeba* spp. adhesion assessment, 200 µL of inoculum to 96-well polystyrene microplates were added, followed by a 48-hour incubation. For co-culture assessment, 100 µL of each inoculum suspension was added, also incubated for 48 hours. After this incubation period, the supernatant was removed to eliminate non-adhered cells, and wells were washed twice with sterile 0.85% saline solution.

To evaluate the adhered cells' total mass, 100 µL of 0.1% crystal violet to the wells for 30 minutes were added. After removing the dye and washing twice with saline solution, the stained adhered cells were solubilized with isopropyl alcohol (Neon®), and absorbance was measured at 570 nm using a TECAN® Infinite M200® microplate reader. This assay was performed in both technical and biological triplicates.

The isolate and the strain of *Acanthamoeba* spp and *C. albicans* were classified as strong biofilm-formers when absorbance values were >0.280 ; medium biofilm-formers: $>0.170-0.279$; weak biofilm-formers: $>0.070-0.170$; and non-biofilm-formers: <0.070 following the methods of de Chaves *et al.* (2020), with some modifications.

2.5 Evaluation of Minimum Antibiofilm Concentration (MAC) in *Acanthamoeba* spp. strain, the clinical isolate, and *Candida albicans*, alone and in co-culture.

In this study, the Minimum Antibiofilm Concentration (MAC) of *Acanthamoeba* spp. and *Candida albicans* both individually and in co-culture were evaluated. Following the incubation of the amoeba and the yeast, the cell counts were performed using a Fuchs-Rosenthal chamber to adjust the inoculum to 8×10^5 cells/mL for *Acanthamoeba* spp. and 8×10^5 CFU/mL for *Candida* spp., utilizing RPMI 1640 medium for both microorganisms. The antifungals and chlorhexidine were prepared at 32 μ g/mL and 200 μ M concentrations, respectively. These concentrations were chosen based on the biological effectiveness of these treatments against *Acanthamoeba* spp. and the Minimum Inhibitory Concentration (MIC) for *C. albicans*, determined according to the M27-A3 protocol by CLSI (2008). The MAC was determined following the methods of Reginatto *et al.* (2022).

For antibiofilm activity evaluation of each organism individually, 100 μ L of the inoculum suspension and 100 μ L of each treatment were added to each well of the microplate. For the co-culture evaluation, 50 μ L of *Acanthamoeba* spp. suspension, 50 μ L of *C. albicans* inoculum suspension, and 100 μ L of each treatment were added to each well, a total volume of 200 μ L per well. The microplates were incubated at 35°C for 48 hours. After incubation, the supernatant was removed, and the wells were washed twice with sterile 0.85% saline solution. Two distinct assays were then performed:

- a) **To evaluate the total mass of adhered cells:** 100 μ L of 0.1% crystal violet was added to the wells and incubated for 30 minutes. After removing the dye, the wells were washed twice with saline solution. The stained adhered cells were then solubilized with isopropyl alcohol (Neon®), and their absorbance was measured at 570 nm using a TECAN® Infinite M200® microplate reader.
- b) **Evaluation of drug susceptibility in adhered cells:** In this assay, Trypan blue dye 1:10 in saline solution was added to the microplate wells. *Acanthamoeba* spp. and *C. albicans* cells were counted using a Fuchs-Rosenthal chamber, with results expressed as cells/mL on a logarithmic scale (Log_{10}). The Trypan blue dye served as a viability indicator, allowing for the quantification of dead cells following treatment and thereby assessing treatment efficacy.

For the growth control, 200 μ L of amoeba and fungal inoculum were added to RPMI 1640 medium. For sterility control, only RPMI 1640 medium was used in the wells. The assays were performed in both technical

and biological triplicates. The Minimum Antibiofilm Concentration (MAC) was defined as the concentration that inhibited more than 80% of the biofilm formation, calculated using specific equations, where OD refers to optical density:

$$\% \text{ Inhibition} = 1 - \frac{[(\text{OD treatment})]}{[(\text{OD growth control})]} \times 100$$

$$\% \text{ Inhibition} = 1 - \frac{[(\text{Viability treatment})]}{[(\text{Viability growth control})]} \times 100$$

2.6 Evaluation of the Minimum Biofilm Eradication Concentration (MBEC)

In this assay, the fungal and amoebic inoculum, both individually and in combination, containing approximately 8×10^4 CFU/mL (*C. albicans*) and 8×10^4 cells/mL (*Acanthamoeba* spp.), were added to the microplates as described in section 2.4. The microplates were incubated at 35°C for 48 hours to allow for biofilm formation. After incubation, the supernatant was removed, and non-adherent cells were washed away with two successive washes using sterile 0.85% saline solution. The antifungals and chlorhexidine were prepared as described in section 2.1, and 100 µL of each were added to the microplates, which were incubated again at 35°C for 48 hours. Following this second incubation, the supernatant was removed, and two successive washes with sterile 0.85% saline solution were performed.

Two parallel assays were conducted as in section 2.5: (a) quantification of the total biomass of adhered cells and (b) assessment of cell viability. Both growth controls (fungal and amoebic inoculum only in RPMI) and negative controls (RPMI only) were included in the experiment. All assays were conducted in triplicate for both technical and biological replicates. The Minimum Biofilm Eradication Concentration (MBEC) was defined as the lowest concentration that could eliminate more than 80% of the formed biofilm (Reginatto *et al.*, 2022), with some modifications. The biofilm removal percentage was calculated using specific equations, where OD refers to optical density:

$$\% \text{ Removal} = 1 - \frac{[(\text{OD treatment})]}{[(\text{OD growth control})]} \times 100$$

$$\% \text{ Removal} = 1 - \frac{[(\text{Viability treatment})]}{[(\text{Viability growth control})]} \times 100$$

2.7 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 9.0 software. One-Way ANOVA followed by the Tukey test was used to evaluate the Minimum Antibiofilm Concentration (MAC) and Minimum Biofilm Eradication Concentration (MBEC).

3. RESULTS

3.1 Evaluation of *Acanthamoeba castellanii* strain, the clinical isolate, and *Candida albicans* adhesion, alone and in co-culture.

To perform co-culture assays, it was necessary to standardize an ideal culture medium for growth and adhesion of the organisms evaluated in this study. *C. albicans*, *Acanthamoeba* spp. strain and clinical isolate exhibited better growth and biofilm formation in RPMI 1640 medium (Figures 1). Thus, all subsequent experiments were conducted in the selected medium.

Assessing the total biomass of adhered cells using crystal violet staining, it was observed that the *C. albicans* strain demonstrated strong biofilm formation, both alone and in co-culture with the strain and with the *Acanthamoeba* clinical isolate. *A. castellanii* and the clinical isolate tested also showed high adhesion capacity. However, when evaluated in co-culture, isolate R40 exhibited lower adhesion (Figure 1).

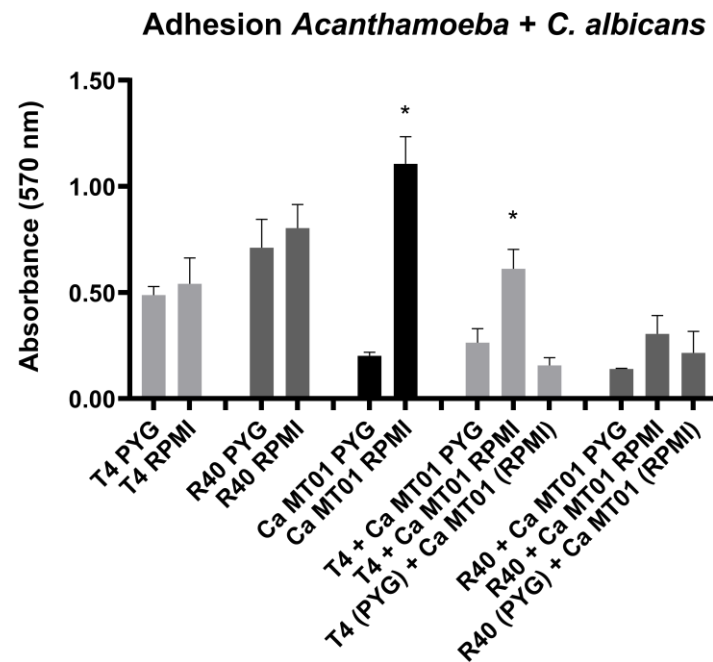


FIGURE 1 Evaluation of *Acanthamoeba castellanii* strain (T4), the clinical isolate (R40) and *C. albicans* (Ca MT01) adhesion, alone and in co-culture. RPMI 1640 and PYG medium were tested. The values expressed were obtained by the arithmetic mean of the absorbances in triplicate, by ANOVA one-way statistical method followed by the Tukey test. The isolate and the strains were classified as strong biofilm-formers when absorbance values were >0.280 ; medium biofilm-formers: >0.170 - 0.279 ; weak biofilm-formers: >0.070 - 0.170 ; and non-biofilm-formers: <0.070 . Statistical significance at $p < 0.05$ is indicated by (*), compared to the control of each evaluated group.

3.2 Evaluation of Minimum Antibiofilm Concentration (MAC) in *Acanthamoeba castellanii* strain, the clinical isolate, and *Candida albicans*, alone and in co-culture.

In evaluating the total biomass of adhered cells following treatment, antifungals and chlorhexidine demonstrated significant antibiofilm activity against all tested organisms, showing a statistically significant difference from the growth control ($p < 0.05$). Natamycin showed slightly superior activity, yielding a higher reduction in the total biomass of adhered cells compared to the other agents. The clinical isolate exhibited increased resistance to treatments. A reduction greater than 80% in the total biomass was observed to voriconazole and natamycin against *A. castellanii* (T4) in co-culture with *C. albicans*, for natamycin against *C. albicans*, and for chlorhexidine against *A. castellanii* T4 (Figure 2a). The assays evaluating *C. albicans* antibiofilm susceptibility demonstrated that the natamycin exhibited superior efficacy, with more than 80% biofilm formation inhibition across all analyses, both individually and in co-culture. The voriconazole also

showed promising results, particularly against the organisms in the co-cultivate situation. Moreover, the chlorhexidine removed almost 100% of the *C. albicans* biofilm in the co-culture assay (Figure 2b).

Based on the antibiofilm susceptibility analysis against *Acanthamoeba* spp., natamycin demonstrated the highest efficacy, achieving 100% biofilm formation inhibition in all evaluations. Chlorhexidine also showed significant activity against the *Acanthamoeba* strain and the clinical isolate, exhibiting over 80% of adhesion inhibition in these organisms (Figure 2c).

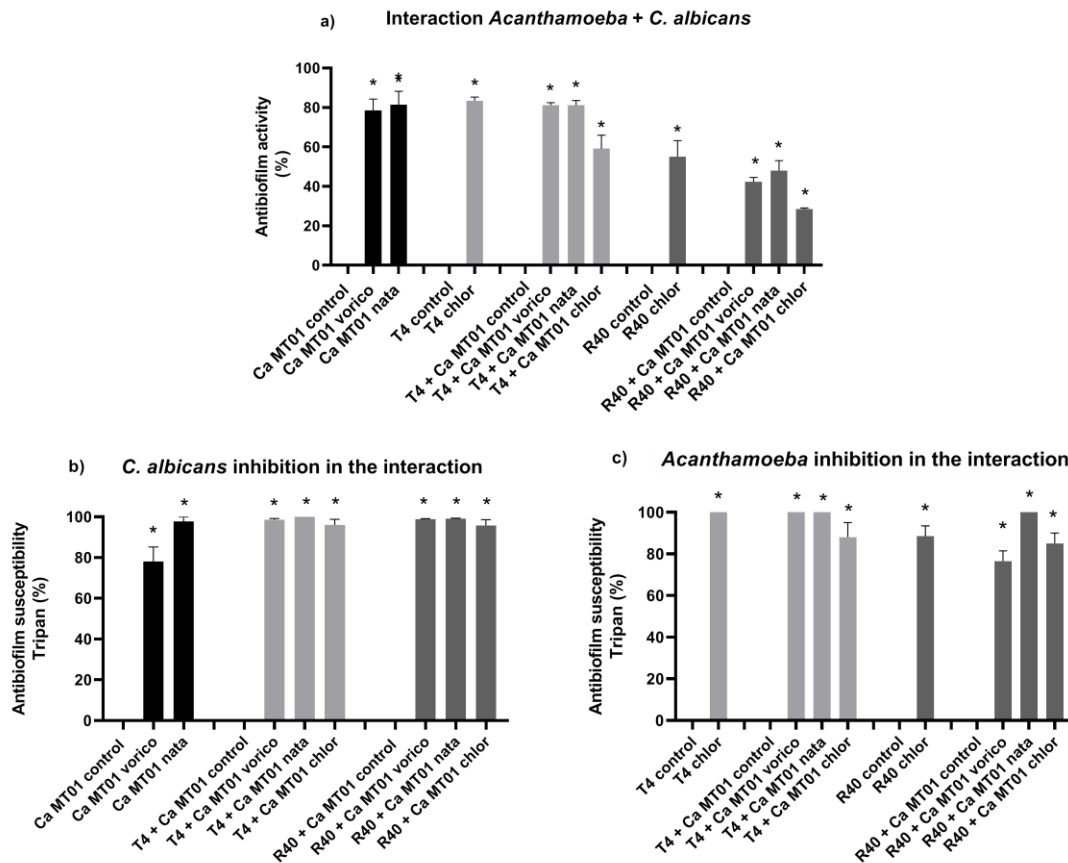


FIGURE 2 Evaluation of the Minimum Antibiofilm Concentration (MAC) for *Acanthamoeba castellanii* strain (T4), the clinical isolate (R40) and *C. albicans* (Ca MT01), alone and in co-culture. (a) the percentage of antifungals antibiofilm activity and chlorhexidine against the organisms alone and in co-culture. In (b) and (c), the percentage of biofilm formation inhibition for *C. albicans* and *Acanthamoeba* are shown, respectively. The strains and the clinical isolate without treatment were used as growth controls. The MAC was defined as the concentration that inhibited more than 80% of biofilm formation. Statistical significance at $p < 0.05$ is indicated by (*), compared to the control of each evaluated group.

3.3 Evaluation of the Minimum Biofilm Eradication Concentration (MBEC)

The antifungals and chlorhexidine demonstrated effective capacity in eliminating biofilm. Among the drugs evaluated, natamycin exhibited the greatest ability to reduce the total biomass of adhered cells in co-

culture assays and against *C. albicans*. Chlorhexidine proved more effective against the *A. castellanii* strain than against the clinical isolate (Figure 3a). Across all analyses, treatment efficacy achieved over 80% of *C. albicans* biofilm eradication. Against *C. albicans* alone, natamycin demonstrated the highest efficacy, achieving complete biofilm eradication (100%) (Figure 3b).

Analyzing the *Acanthamoeba* removal in the biofilm after treatment application, was observed that natamycin showed 100% elimination of the biofilm formed in all analyses, with a statistically significant difference from the growth control ($p < 0.05$). Voriconazole demonstrated over 80% biofilm eradication in co-culture assays. Furthermore, chlorhexidine showed promising results, achieving 100% elimination of both the *Acanthamoeba* strain and clinical isolate (Figure 3c).

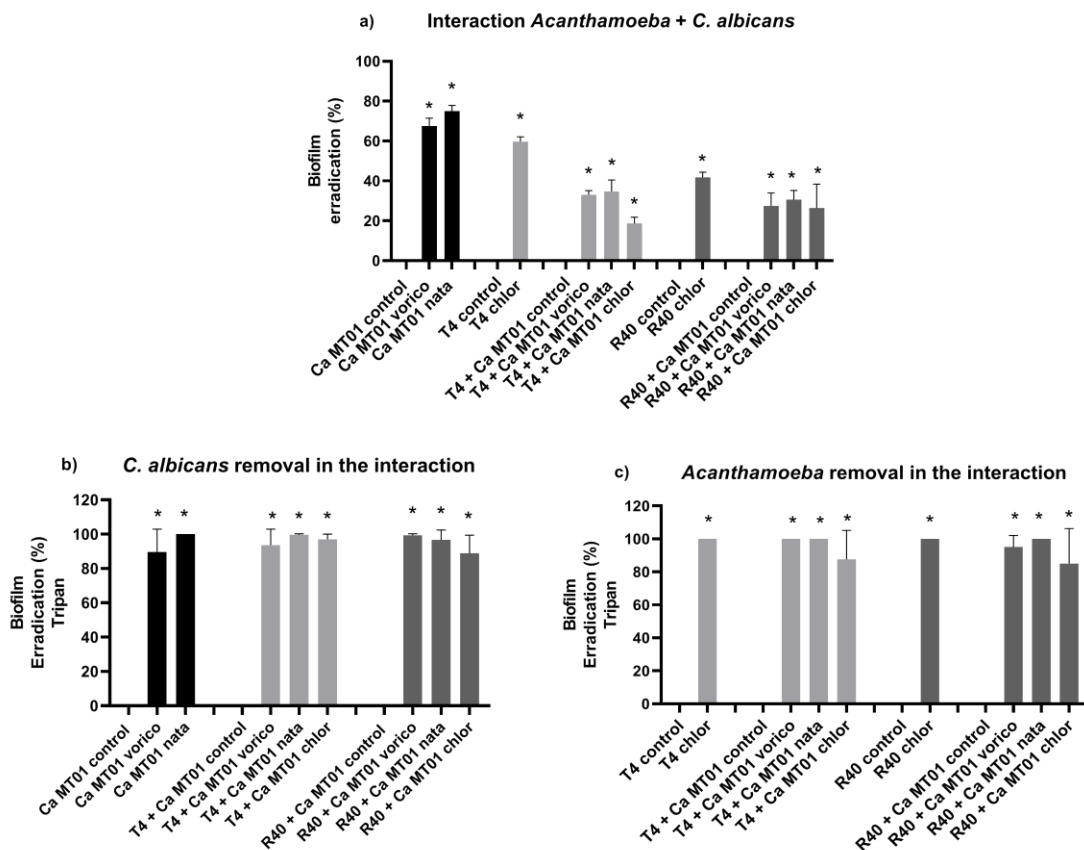


FIGURE 3 Evaluation of the Minimum Biofilm Eradication Concentration (MBEC) for *Acanthamoeba castellanii* strain (T4), the clinical isolate (R40) and *C. albicans* (Ca MT01), alone and in co-culture. (a), percentage of biofilm eradication activity by antifungals and chlorhexidine against the organisms alone and in co-culture. In (b) and (c), the percentage *C. albicans* and *Acanthamoeba* removal from the formed biofilm is presented, respectively. Each strain without treatment was used as a negative control. The MBEC was defined as the lowest concentration that could eliminate more than 80% of the formed biofilm. Statistical significance at $p < 0.05$ is indicated by (*), compared to the control of each evaluated group.

4. DISCUSSION

This study standardized methods for investigating the adhesion capacity of *Acanthamoeba* spp. strain and clinical isolate, alone and in co-culture with *C. albicans* and anti-biofilm and biofilm eradicating susceptibility of voriconazole, natamycin, and chlorhexidine. These drugs are widely used at polymicrobial keratitis. The RPMI 1640 medium was selected for *C. albicans* and *Acanthamoeba* spp. co-culture assays due to its efficiency in supporting these organisms growth and biofilm formation. Although the PYG medium is the standard recommendation for cultivating *Acanthamoeba* spp. (Eroğlu *et al.*, 2015), our results showed that *C. albicans* did not show good adhesion capacity and biofilm formation in this medium (Figure 1). Furthermore, *Acanthamoeba* demonstrated strong adhesion in the RPMI 1640, agree with the findings by Eroğlu *et al.* (2015), in which RPMI was the most rapid and convenient medium for the growth of *Acanthamoeba* spp.

Adhesion was observed for both the strain, and *Acanthamoeba* spp. clinical isolate in 96-well microplates (Figure 1). These results support existing literature that highlights the strong adhesion capacity of *Acanthamoeba* spp. in polystyrene plastic surfaces (Mitsuwan *et al.*, 2020) and contact lenses (Lee *et al.*, 2018; Mitsuwan *et al.*, 2020; Pinto *et al.*, 2024). Furthermore, the clinical isolate R40 showed a greater adhesion capacity compared to the *A. castellanii* strain (ATCC 50492), consistent with previous *in vitro* studies that have reported increased adhesion of clinical isolates to contact lenses (Pinto *et al.*, 2024). This adhesion profile of clinical isolates may be associated with the ability of *Acanthamoeba* spp. to carry pathogenic microorganisms, such as bacterial within its trophozoite and cyst structures, increasing its virulence and consequently, adhesion to surfaces, as well as the high pathogenic potential of the clinical isolate evaluated (Marciano-Cabral *et al.*, 2003).

C. albicans and *Acanthamoeba* spp. co-culture assays, there was an accentuated reduction in the total adhered biomass compared to the biomass of *C. albicans* cultured alone (Figure 1). Literature indicates that the biofilm biomass produced by MRSA and *M. bovis* was significantly diminished when co-incubated with *A. castellanii*. This phenomenon has been explained by the amoeba's ability to release substances that disperse the established biofilm (Martin *et al.*, 2020).

Candida species biofilm formation represents a significant public health challenge, particularly in severe infectious keratitis cases (Reginatto *et al.*, 2023). Various pathogens, including *Acanthamoeba* spp., can inhabit this complex structure to enhance resistance to clinical treatments (Pinto *et al.*, 2021). A study conducted in Iran identified *Acanthamoeba* in 26.08% of domestic biofilm samples and highlighted the potential risk of *Acanthamoeba* keratitis in individuals who wear soft contact lenses (Norouzi *et al.*, 2021). The available therapeutic options are limited and present several constraints (Ting *et al.*, 2021).

Natamycin, a polyene macrolide antifungal, is effective against yeasts by irreversibly binding to ergosterol, a crucial component of fungal cell membranes (Agustín *et al.*, 2023). Voriconazole, an antifungal belonging to the triazole class, acts by inhibiting ergosterol synthesis, thereby destabilizing the fungal membrane (Herrick; Patel; Hashmi, 2024). Chlorhexidine, on the other hand, is a well-known antiseptic and disinfectant agent recognized for its antibacterial and antifungal properties (Łukomska-Szymańska; Sokołowski; Łapińska, 2017). Both antifungals and chlorhexidine are widely used for the polymicrobial keratitis treatment (Kosrirukvongs; Wanachiwanawin; Visvesvara, 1999; Lim *et al.*, 2008; Prajna *et al.*, 2010; Bang *et al.*, 2010; Sun *et al.*, 2014; Buchele *et al.*, 2018). However, there is limited research evaluating the effectiveness of these drugs in biofilm formation and eradication, particularly those involving *Acanthamoeba* spp.

A recent study demonstrated that natamycin caused a significant disruption in the *C. tropicalis* biofilms at high concentrations. Nevertheless, its action against the polymicrobial biofilms has not been assessed (Agustín *et al.*, 2019). In this study, natamycin treatment effectively reduced the *C. albicans* biofilm formation, while the treatment with both, voriconazole and natamycin, significantly decreased *A. castellanii* (T4) biofilm formation in co-culture with *C. albicans*. This resulted in more than 80% reduction in the total adhered biomass. Additionally, chlorhexidine was able to remove almost 100% *C. albicans* biofilm in the co-culture assay (Figure 2a). In agree a previous *in vitro* study by Landa *et al.* (2024) has shown the antimicrobial potential of chlorhexidine against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms.

It is also important to highlight the resistance increased in the *Acanthamoeba* clinical isolate R40 to the treatments (Figure 2a), which corroborates with the findings by Megha *et al.* (2022). In their study, the *Acanthamoeba* clinical isolates exhibited higher minimum inhibitory concentrations (MICs) for polymyxin B and fluconazole when compared to the environmental isolates, indicating greater resistance. This resistance profile may be associated with the high clinical isolates virulence potential, which are often previously exposed to treatments aimed at eradicating keratitis, thus enhancing their resistance over time.

C. albicans biofilms are less susceptible to antifungal agents, primarily due to the challenges in reducing metabolic activity and to resistance that these microorganisms confer to drugs. According to Chandra *et al.* (2015), these biofilms consist of a complex network of yeast cells, hyphae, and pseudo-hyphae, forming multiple layers. Building on this, this study demonstrated over 80% efficacy in *C. albicans*, both individually and in co-culture, against natamycin. Voriconazole and chlorhexidine also showed significant efficacy, achieving over 80% inhibition of the *C. albicans* biofilm formation in co-culture (Figure 2b). The effective action of these

drugs becomes even more apparent when compared to results from a previous study on antifungal agents, such as amphotericin B, against *Candida* spp. biofilms (Reginatto *et al.*, 2020).

The susceptibility profile evaluation of *Acanthamoeba* biofilms has not been reported in the literature. A study has described that the amino acids arginine and lysine, when incorporated into the structure of rhamnolipids, achieved significant antifungal, antibiofilm, and amoebicidal activities. In this study, *Candida* spp. and *Acanthamoeba castellanii* strains are fluconazole-resistant for use for the assays (da Silva *et al.*, 2020). In this regard, we investigated the ability of antifungal and chlorhexidine to inhibit *Acanthamoeba* spp. biofilm formation in co-culture with *C. albicans*. Natamycin demonstrated the highest efficacy, achieving 100% inhibition of *Acanthamoeba* spp. Additionally, chlorhexidine showed a significant effect against both the strain and the clinical isolate of *Acanthamoeba* spp., individually and in co-culture, with more than 80% inhibition of adhesion by this organism (Figure 2c).

Regarding the evaluation of the MBEC, it is important to emphasize that the microorganisms exhibited greater resistance to tested concentrations (Figure 3a). This resistance is directly linked to the biofilm formation process. In these assays, natamycin demonstrated the highest ability to reduce adhered biomass in co-culture assays and against *C. albicans*. Chlorhexidine proved to be more effective against the *Acanthamoeba* strain, showing a similar profile to the antibiofilm activity assays (Figure 3a). Both antifungals and chlorhexidine exhibited excellent biofilm eradication activity, achieving over 80% *C. albicans* biofilm elimination (Figure 3b). The antifungals ability to remove pre-formed *C. albicans* biofilm in polyurethane central venous catheters was evaluated in the study by Reginatto *et al.* (2020), in which amphotericin B and fluconazole achieved between 78% and 100% biofilm eradication, similar to the results obtained with the antifungals tested in our study. Analyzing the effectiveness of the treatments, all the tested treatments demonstrated high efficacy against *Acanthamoeba* spp., with more than 70% biofilm eradication. Natamycin stood out, achieving 100% elimination of *Acanthamoeba* in all analyses (Figure 3c).

Our findings underscore the significant challenge about *Acanthamoeba* adhesion and biofilm formation in polymicrobial infections, particularly in the context of co-infections with *C. albicans*. *Acanthamoeba* strong adhesion capacity enhances its persistence and resistance to treatment, highlighting the importance of addressing both fungal and amoebic components in therapeutic strategies. Natamycin, voriconazole, and chlorhexidine are highly effective in biofilm eradication, with natamycin showing the greatest efficacy, particularly in co-culture assays. Notably, *Acanthamoeba* clinical isolate exhibited increased resistance, reinforcing the need for more targeted and robust treatment approaches for these biofilms. The amoeba adhesion evaluation is particularly

important, as it helps to better understand the pathogenic mechanisms involved, which can lead to more effective and targeted therapeutic approaches in combating these challenging infections. So, this study provides valuable insights into developing more effective strategies for treating and preventing polymicrobial keratitis. Additionally, the methodologies developed can be applied to evaluate the efficacy of multipurpose solutions for disinfecting contact lens cases and lenses, as well as maintaining these materials by inhibiting biofilm formation.

5. CONCLUSION

This study is unprecedented and highlights the standardization of different methodologies to evaluate the *Acanthamoeba* in co-cultivation with *C. albicans* adhesion capacity. Thus, it was possible to observe that both, the ATCC strain and the *Acanthamoeba* spp. R40 isolate showed adhesion potential on polystyrene microplates, alone and in co-culture with *C. albicans*. Antibiofilm efficacy trials and eradicating potential of different drugs were evaluated. Natamycin, voriconazole, and chlorhexidine showed promising activity in anti-biofilm and biofilm eradicating against *Acanthamoeba* strain, the clinical isolate and *C. albicans*. Among the antifungals, natamycin exhibited the greatest efficacy against *C. albicans* biofilm both individually and in co-culture with *Acanthamoeba*. Chlorhexidine proved to be particularly effective, achieving *Acanthamoeba* 100% eradication, highlighting its potential for treating *Acanthamoeba* related infections. The *Acanthamoeba* R40 clinical isolate demonstrated greater resistance compared to the ATCC strain, because it may exhibit enhanced virulence and resistance profiles. So, this study emphasizes the importance of further research into optimizing antibiofilm and biofilm eradicating treatments for improved clinical outcomes in managing infections caused by both *Acanthamoeba* and *C. albicans*. A better understanding of the *Acanthamoeba* spp. adhesion capacity alone or in association with *C. albicans* will assist in the search for better treatment strategies and the microorganisms behaviors in cases of severe polymicrobial keratitis. Moreover, the standardized methods developed in this study can be applied to evaluate the efficacy of multipurpose solutions for disinfecting and maintaining contact lens cases and lenses by inhibiting biofilm formation, ultimately improving their safety, performance, and effectiveness in preventing infections.

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Conflicts of Interest

The authors declare no conflicts of interest.

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