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ACTIVITY OF CHLORHEXIDINE ASSOCIATED WITH SILVER NANOPARTICLES AGAINST *Candida albicans* BIOFILMS ON TOOTHBRUSHES

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ABSTRACT

The yeast *Candida albicans* is a normal constituent of the human microbiota with considerable pathogenicity and ability to form microbial biofilms in various anatomical sites, including the oral cavity. Chlorhexidine is an antiseptic widely used against Gram-positive and Gram-negative bacteria, fungi, yeasts, and lipophilic viruses. Silver nanoparticles (AgNPs) have shown potential antiseptic effects against yeasts. In this context, this study aimed to evaluate the activity of chlorhexidine gluconate and silver nanoparticles against *Candida albicans* ATCC 10231 inoculated on sterilized toothbrushes. Previously inoculated toothbrushes were exposed to the compounds at room temperature for 20 minutes for the initial 1-hour adhesion test, and for 1 hour against mature biofilms cultured for 48 hours at 35.5 °C, then the samples were diluted and plaqueted for the quantification of viable yeasts after treatment. The most significant reduction in yeast viability was observed with the combination of chlorhexidine and AgNPs, with 97.2 % in the initial adherence test and 77.2 % against biofilms. This finding suggests a synergistic effect between the compounds against *Candida albicans*, with a more pronounced effect against the planktonic state than against mature biofilms.

Keywords: Antiseptics; AgNP; Yeast; Adherence.

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INTRODUCTION

Oral health influences quality of life, and oral health disorders are considered public health problems because although they are preventable and known to be controlled, they tend to affect more vulnerable individuals (PERES et al., 2019; PROSBT et al., 2019). A diversified oral microbiota is essential for maintaining good oral and systemic health and should consist of communities of bacteria, fungi, and viruses that reside in different niches of the oral cavity. However, using antiseptics can result in a less diverse dysbiotic microbiome (ROHR et al., 2021).

The oral microbiota is complex. Although a predominant bacteria is involved in extensive biofilm formation, yeasts of the genus *Candida* are frequent microbiota members. Although they do not present pathological risks in healthy individuals, they are considered a species with pathogenic potential, as they are also excellent biofilm formers (BAUMGARDNER, 2019; SANTANA et al., 2013).

Hygiene is necessary for maintaining oral health, as microbial biofilm formation can cause or aggravate periodontal diseases, caries, and different infections by various microbial species (DE MENEZES et al., 2020). Toothbrushing is one of the most widely used methods of oral hygiene. However, by removing microbial biofilm and other debris during brushing, toothbrushes are contaminated with bacteria, blood, saliva, and oral debris and can be a source of infection (NAIK et al., 2015).

Brushing and flossing help remove microorganisms mechanically adhering to oral surfaces. They are fundamental strategies for maintaining oral hygiene when using antiseptic solutions for mouthwashes. In the dental context, chlorhexidine is used as a mouthwash and is often applied during dental care to reduce the oral bacterial microbiota. In addition, it can be used to prevent and treat diseases related to dental biofilms, such as some forms of gingivitis or periodontitis (FIORILLO, 2019). Chlorhexidine is one of the most widely used skin antiseptics due to its efficacy and a broad spectrum of action. It is active against Gram-positive and Gram-negative bacteria, viruses, and fungi (BROOKES et al., 2020).

Developing new antiseptics has been challenging due to microbial resistance to several antibiotics. An alternative could be associating known antiseptics with nanostructured materials such as silver nanoparticles (AgNPs). Although the antimicrobial activity of silver has a long history in medicine, AgNPs are currently gaining increasing prominence as antifungal agents due to their broad spectrum of antimicrobial activity and their enormous number of applications in health sciences, from topical formulations to catheters impregnated with AgNPs (MUSSIN & GIUSIANO, 2022).

Nanomaterials represent an innovative class of antimicrobial substances. Silver nanoparticles have been investigated as attractive options as antiseptics due to their physical, chemical, and biological properties (CRISAN et al., 2021; RADHAKRISHNAN et al., 2016; HADI et al., 2024). Due to their anti-caries, anti-biofilm and anti-inflammatory properties, AgNPs have been evaluated in dental biomaterials, acrylic resin used in dental prostheses, as well as composite resins, orthodontic adhesives and cements, titanium implants, porcelain restorations and endodontic materials (MALLINENI et al., 2023).

A recent systematic review provided an overview of toothbrush contamination and the factors affecting toothbrush contamination with an evidence-based approach. The main conclusions were that toothbrush contamination begins immediately after first use, that there is a direct correlation with prolonged use and an inverse relationship with drying time. Strategies such as the use of charcoal, green tea extract, nanogold-coated bristles and post-brushing rinsing with chlorhexidine-based mouthwash are effective measures to reduce contamination (KHAN et al., 2024).

Used toothbrushes serve as reservoirs for microorganisms and can play an important role in the transmission of diseases in humans. It is therefore recommended that everyone in daily life disinfects their toothbrushes between uses and stores them in a clean, dry place separately to optimize oral hygiene and systemic health (TON et al., 2022).

This study evaluated the activity of chlorhexidine gluconate and silver nanoparticles sprayed on toothbrushes inoculated with *Candida albicans* ATCC 10231 after 1 hour of incubation to analyze the effect of the compounds on the initial adherence of the yeasts and against mature biofilms grown at 35.5 ºC after 48 hours of incubation.

MATERIALS AND METHODS

Compounds evaluated

The Dental Fresh Periotrat mouthwash (L: 215261 V: 09/2024) manufactured by Kley Hertz Farmacêutica S.A. Porto Alegre - RS was used consisting of 0.12 %

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chlorhexidine gluconate and silver nanoparticles (AgNP) synthesized at the Materials Chemistry and Molecular Modeling Laboratory.

AgNPs were synthesized by adapting the methodology (KERESELIDZE et al, 2012). Briefly, the synthesis began with adding 5 mL of sodium citrate solution (5.4 mol L^{-1}) in 200 mL of $AgNO_3$ solution (0.24 mmol L^{-1}), with the mixture being kept under magnetic stirring for 15 minutes (at room temperature). Next, 8 mL of sodium borohydride solution (40 mmol L^{-1}), previously prepared and stored under refrigeration, was added to the reaction mixture. This mixture remained under magnetic stirring for 20 minutes. After this, the magnetic stirring was stopped, and the reaction mixture was uncovered in the dark for 2 hours. Finally, the colloid obtained was sterilized in an autoclave at 121 °C for 15 min. The concentration of silver ions was determined using atomic absorption spectroscopy in Perkin Elmer's AAnalyst 400 equipment; the value determined was 0.082 mg mL^{-1}.

Microbiological tests

The microbiological tests were conducted at the Bioassay Laboratory of the Research and Postgraduate Center of the State University of Goiás (UEG). Disposable toothbrushes (Figure 1) with a size of 147 mm, a 25 mm head, 23 tufts, 648 white and 180 green bristles, totaling 828 nylon bristles, were used.

Figure 1. The aspect of the toothbrushes used in the study.

The tests were performed by inoculating *Candida albican*s ATCC 10231 onto the previously sterilized toothbrushes to assess initial adherence and biofilm formation. The yeast colonies grown on Sabouraud agar were dissolved in a sterile physiological solution (SPS). The turbidity of the solution was adjusted using the 0.5 McFarland scale, and the inoculum was diluted to 1/100 by transferring 0.15 mL of the microbial suspension to a conical tube containing the toothbrush and 14.85 mL of Sabouraud broth.

The inoculated toothbrushes were kept at room temperature for one hour for the initial adherence samples. At the same time, they were incubated in an oven at 35.5 °C for 48 h for the biofilm formation test (Figure 2).

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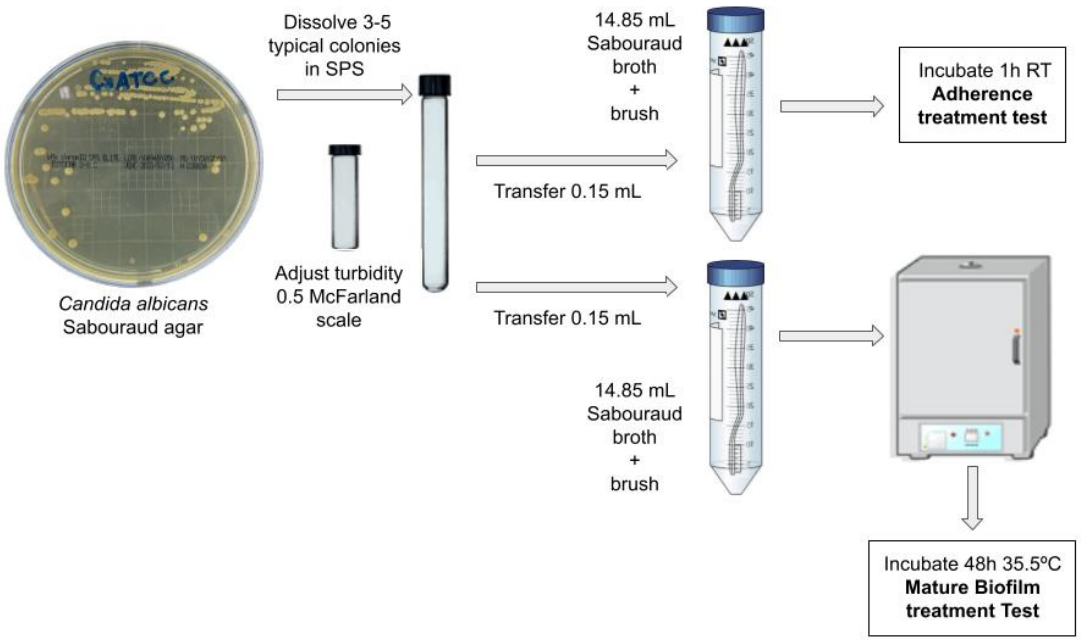
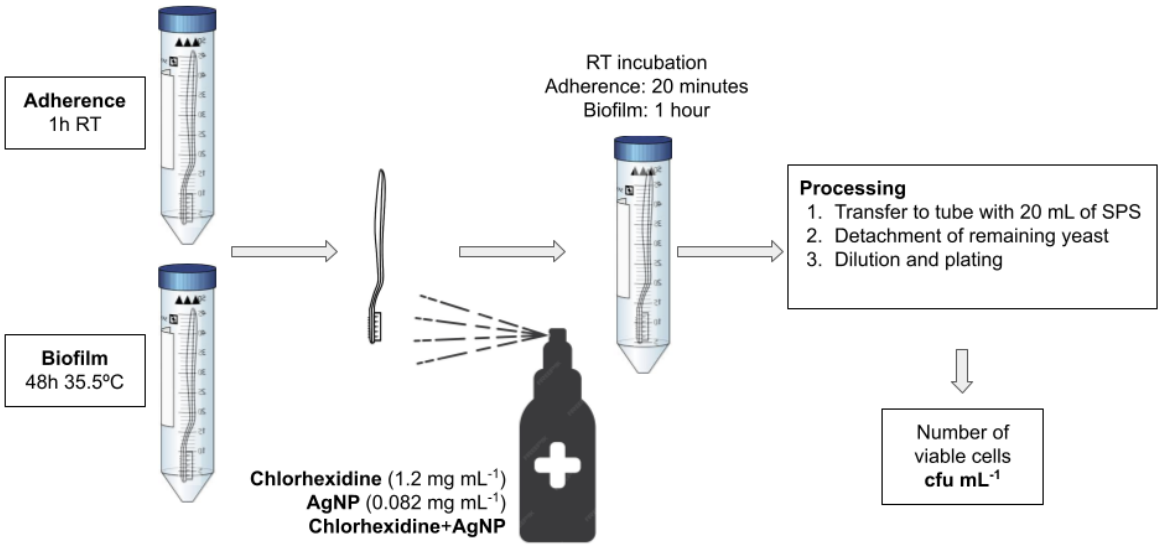
Figure 2. 1-hour initial adherence and 48-hour biofilm formation tests of *C. albicans* ATCC 10231 on toothbrushes.



After incubation, the toothbrushes were aseptically processed by removing them from the culture broths, rinsing them in SPS and spraying the toothbrush heads with 435 ± 93 µL of chlorhexidine solution and colloidal AgNP dispersion alone and in combination of both (Figure 3).

Figure 3. Processing of toothbrushes after incubations.



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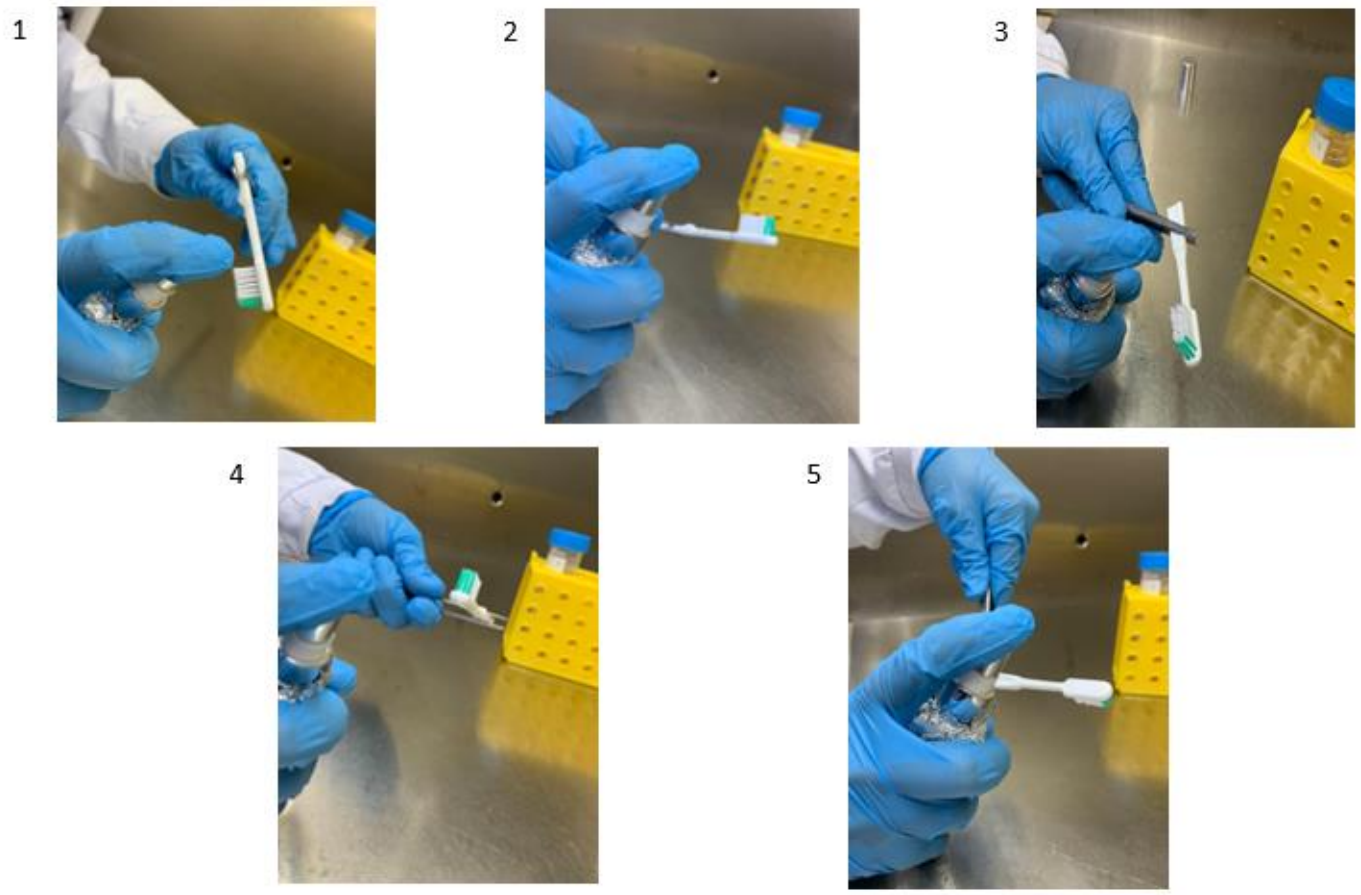
The sprayed toothbrushes were kept in contact with the compounds for 20 minutes at room temperature for the initial adhesion inhibition test and for one hour for the toothbrushes with a 48-hour mature biofilm (Figure 4).

Figure 4. Spraying the toothbrush with chlorhexidine gluconate solution 1.2 mg mL^{-1} and AgNP colloidal dispersion 0.082 mg mL^{-1}.



1. Application of the compounds to the side of the toothbrush; **2.** Application to the other side; **3.** Application to the front; **4.** Application to the toothbrush head; **5.** Application to the back of the toothbrush.

The pulverized toothbrushes were then transferred to conical tubes containing 15 mL of SPS and shaken in a vortex for one minute to loosen the adhered viable yeasts. Afterward, the toothbrushes were discarded, and the SPS was diluted and plated to count the viable yeasts (cfu mL^{-1}) on Sabouraud agar.

In addition to the treated toothbrushes, unsprayed inoculated toothbrushes were included as a yeast viability control, and the initial inocula was quantified. All tests were carried out in three independent replicates and the results were used to calculate means and standard deviations.

RESULTS AND DISCUSSION

The counts of viable yeasts adhering to toothbrushes not sprayed with the compounds are shown in Table 1. The initial inoculums for all the tests were 9.70 x 10^3 ± 2.97 x 10^3 cfu mL^{-1}.

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Table 1. Viable yeast counts (cfu mL^{-1}) in the adherence and biofilm formation tests on the toothbrushes.

Test Conditions	Mean	Standard Deviation
Initial inoculum	**9.70×10^3**	$\pm 2.97 \times 10^3$
Adherence 1h	**1.25×10^3**	$\pm 4.24 \times 10^2$
Biofilm 48h	**3.09×10^5**	$\pm 2.20 \times 10^5$

C. albicans ATCC 10231 could adhere to the toothbrushes with counts of $1.25 \times 10^3 \pm 4.24 \times 10^2$ cfu mL^{-1} after a one-hour incubation period. The number of cells associated with the mature biofilm after 48 h of incubation rose to $3.09 \times 10^5 \pm 2.20 \times 10^5$ cfu mL^{-1}, demonstrating that the yeasts were capable of forming biofilm under the experimental conditions of this study.

Notwithstanding, the toothbrush is contaminated after the first use, and with continued use, the level of contamination increases. Humidity in the storage area, the use of a plastic cap, the shape of the toothbrush, and the type of bristles tend to increase contamination. While the use of chlorhexidine-based mouthwash significantly reduces the contamination of toothbrushes (KHAN et al., 2024).

However, in our study, we found that *C. albicans* was less sensitive to chlorhexidine when associated with biofilms on the toothbrushes and that, interestingly, the silver nanoparticle was more active against the biofilm than the yeasts during the initial adherence phase on the toothbrushes.

C. albicans can form a complex and resistant biofilm on the surface of dental materials, constituting the main etiological factor of prosthetic stomatitis that damages the oral mucosa. Nevertheless, there is no consensus in the literature on the best method to prevent or combat biofilms on the surface of these materials (MORAES et al., 2021).

Several studies have shown that the environment within a fungal biofilm, including the morphological variation within this structure (yeasts, pseudohyphae, and hyphae), confers advantages over free-living planktonic growth (AIRES et al., 2021; PONDE et al., 2021).

The material type is one factor that influences yeast adhesion and subsequent biofilm development. One of the main components of toothbrush fibers is nylon, a thermoplastic material from the polyamide family (PONDE et al., 2021). Various synthetic materials have been tested, and the most adherent to *C. albicans* were latex, silicon, elastomer, and Teflon (MALINOVSKÁ et al., 2023). In our study, *C. albicans* ATCC 10231 could adhere and form biofilm on toothbrush surfaces.

In the initial adherence test, chlorhexidine significantly reduced viable yeasts (94.1 %) after 20 minutes of exposure to the inoculated toothbrushes. Silver nanoparticles were also active against adhered yeasts but with a lower inhibitory effect, with a reduction of 23.0 %. However, the associated compounds showed increased inhibitory activity, inhibiting 97.2 % of the yeasts adhered to the toothbrushes (Table 2).

Table 2. Counts (cfu mL^{-1}) and percentage reduction of viable yeasts adhered to the sprayed toothbrushes.

Sprayed toothbrushes	Adherence - 1h			Biofilm - 48h		
	Mean	SD	*% reduction*	**Mean**	SD	*% reduction*
Chlorhexidine	**7.33×10^1**	$\pm 2.08 \times 10^1$	*94.1*	**2.54×10^5**	$\pm 3.89 \times 10^4$	*17.9*
AgNP	**9.60×10^2**	$\pm 4.50 \times 10^2$	*23.0*	**1.30×10^5**	$\pm 3.54 \times 10^3$	*58.0*
Chlorhexidine + AgNP	**3.50×10^1**	$\pm 2.12 \times 10^1$	*97.2*	**7.05×10^4**	$\pm 1.06 \times 10^4$	*77.2*

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However, the compounds performed differently against biofilm-associated yeasts. The action of chlorhexidine was reduced to 17.9 %, and the activity of silver nanoparticles was significantly greater against biofilm-associated cells, reducing cell viability by 58.0 % compared to untreated toothbrushes. The inhibitory activity of the combination of chlorhexidine and AgNP was 97.2 % before biofilm formation and 77.2 % after biofilm formation.

Another study found that chlorhexidine 1.2 mg mL^{-1} reduced 95.8 % of adhered *C. albicans* inoculated onto acrylic material for two hours at 37 ºC (GHAZAL et al., 2019). In our study, the percentage of inhibition by chlorhexidine was similar.

Chlorhexidine is one of the most potent antiseptics used in the clinic. Although its mechanism of action is not fully understood, some evidence suggests that it is related to increased cell membrane permeability, which causes penetration of the antiseptic, disruption of ionic homeostasis, damage, and cell death (JIANG et al., 2023).

From a structural point of view, chlorhexidine is a bisbiguanide made up of four chlorophenyl rings and two biguanide groups connected by a central hexamethylene bridge. In addition, it has a strongly basic character with pH levels > 3.5 and positive charges on both sides of the hexamethylene bridge (THANGAVELU et al., 2020).

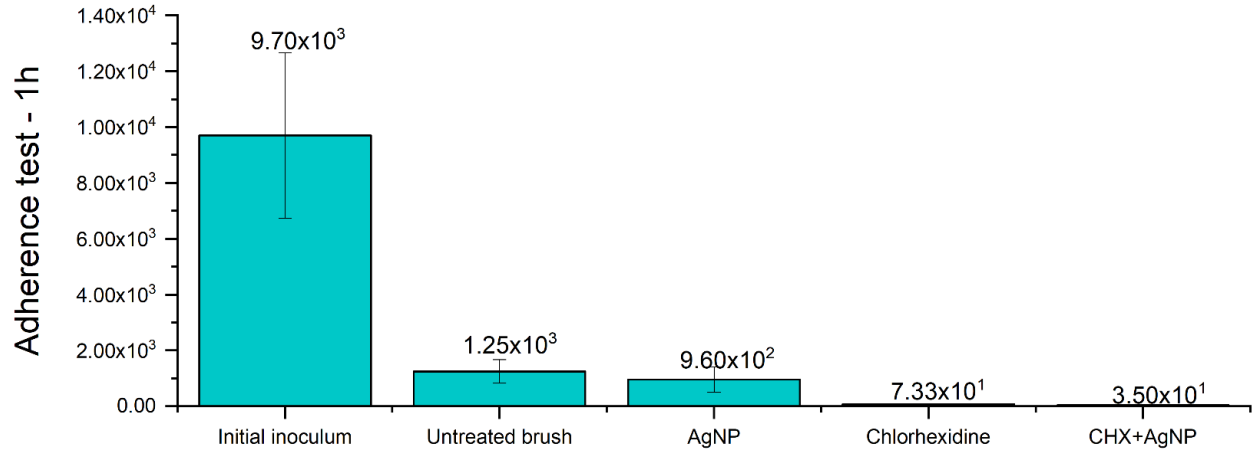
In our study, silver nanoparticles showed less inhibitory activity, inhibiting 23.0 % of the yeasts compared to the viability control. In combination, the two compounds could inhibit 97.2 % of the adhered yeasts in one hour of incubation.

The cellular mechanisms of the antibacterial action of silver nanoparticles are related to (1) physical interaction with the cell membrane, disruption of the lipid bilayer and/or release of cationic ions, (2) contact and reaction of cationic silver with bacterial DNA, resulting in structural alterations, (3) interaction with cytoplasmic structures, including ribosomal protein synthesis machinery, causing translation blockage and reduced transcription and protein synthesis and (4) formation of reactive oxygen species resulting in bactericidal action based on oxidative stress (CRISAN et al., 2021).

Figure 5 shows the number of viable yeasts recovered before and after treatment in the one-hour adherence method and biofilm formation after 48 hours

Figure 5. Viable yeast count (cfu mL^{-1}) in the different tested conditions. A) Initial Adherence, and B) Mature biofilm

A)



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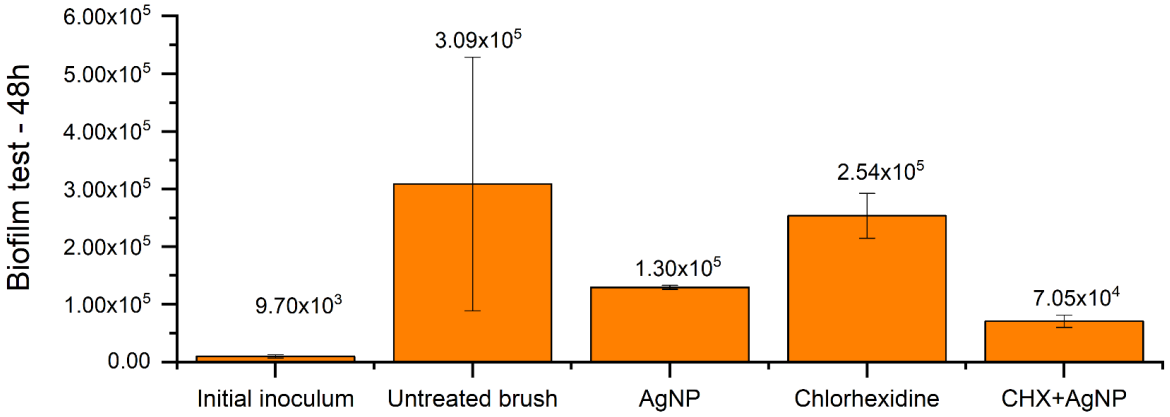
B)



AgNP - Silver nanoparticle 0.082 mg mL^{-1}, CHX+AgNP - Association of chlorhexidine 1.2 mg mL^{-1} and silver nanoparticle 0.082 mg mL^{-1}

The activity of AgNP was more significant against the *C. albicans* biofilm, with a 58.0 % reduction in viability, than against the yeasts in the initial adherence (23.0 %). This result may have been because the exposure time to AgNP was 20 minutes for the initial adherence test and one hour for the mature biofilm. Our results are divergent from a previous study, which found that exposure to AgNPs was less effective in reducing the biomass of the *C. albicans* biofilm and the number of viable cells associated with pre-formed biofilms than when applied to adhered yeasts (MONTEIRO et al., 2011).

In addition, the antifungal activity of nanoparticles against *C. albicans* has been shown to affect adhesion and the development of hyphae, which are crucial steps in yeast pathogenesis, and to inhibit biofilm formation (SLAVIN et al., 2022).

CONCLUSION

Nanotechnology is considered one of the most promising areas with biotechnological applications. Nanoparticles have a variety of biomedical applications as nanostructured materials with varied shapes and sizes and antimicrobial activity (KAUSHAL et al., 2023).

In our study, we found that the combination of chlorhexidine and AgNP was more active in the initial adherence stage, reducing 97.2 % of yeast viability. However, it also performed well against mature yeast biofilms, reducing cell viability by 77.2 %. This finding suggests a synergistic effect between the compounds against *Candida albicans*, with a more pronounced effect against the planktonic state than against mature biofilms. Within the limitations of this study, future randomized studies are recommended to evaluate the effect of different strategies for inhibiting the formation of microbial biofilms, which, once established, contaminate toothbrushes and are difficult to eradicate.

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