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Silver colloidal nanoparticle stability: influence on *Candida* biofilms formed on denture acrylic

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Abstract

Our aim in this study was to evaluate how the chemical stability of silver nanoparticles (SNs) influences their efficacy against *Candida albicans* and *C. glabrata* biofilms. Several parameters of SN stability were tested, namely, temperature (50°C, 70°C, and 100°C), pH (5.0 and 9.0), and time of contact (5 h and 24 h) with biofilms. The control was defined as SNs without temperature treatment, pH 7, and 24 h of contact. These colloidal suspensions at 54 mg/L were used to treat mature *Candida* biofilms (48 h) formed on acrylic. Their efficacy was determined by total biomass and colony-forming unit quantification. Data were analyzed using analysis of variance and the Bonferroni post hoc test ($\alpha = 0.05$). The temperature and pH variations of SNs did not affect their efficacy against the viable cells of *Candida* biofilms ($P > 0.05$). Moreover, the treatment periods were not decisive in terms of the susceptibility of *Candida* biofilms to SNs. These findings provide an important advantage of SNs that may be useful in the treatment of *Candida*-associated denture stomatitis.

Key words: silver nanoparticles, chemical stability, biofilms, *Candida albicans*, *Candida glabrata*.

Introduction

Candida-associated denture stomatitis is frequent in elderly people, especially those who wear complete dentures, and is one of the most common forms of oral candidiasis [1,2]. Even though *Candida albicans* is strongly associated with the development of denture stomatitis, non-*C. albicans Candida* species, such as *C. glabrata*, may also contribute to this pathological condition [3,4]. *Candida* species form biofilms on acrylic denture surfaces that

are characterized by networks of yeasts, pseudohyphae, and hyphae surrounded by an extracellular matrix [5] and embedded mainly into irregularities of acrylic surfaces [6]. Unlike *C. albicans*, *C. glabrata* do not form hyphae [7].

Although antifungal suspensions that are based on nystatin, amphotericin B, miconazole, and fluconazole have been widely used to treat denture stomatitis [8,9], *Candida* infections are frequently recalcitrant to conventional treatments [1,10–12]. Indeed, established *Candida* biofilms are more resistant to antimicrobial agents than their planktonic counterparts [13,14]. This serious clinical impact of increased resistance to conventional antifungal therapy has strengthened the search for alternative strategies that could contribute significantly to the prevention and destruction of *Candida* biofilm formation. For this purpose, silver nanoparticles (SNs) are recognized as a new class of antimicrobial agents [15] because of their effective antimicrobial ability. SNs have a large surface area and high reactivity compared with microparticles [16]. In dentistry, these nanoparticles have been incorporated into dental adhesives [17,18] and resin composites [19] in order to inhibit dental caries and into denture base acrylic resin [20] in order to prevent stomatitis.

The mechanisms of killing microorganisms of SNs are multifactorial [15,16,21–23], and it is believed that these mechanisms are similar to those of silver ions (SIs) [21,24]. In general, SNs bind to sulfur-containing proteins in biological molecules, resulting in defects in the microbial cell membrane and loss of intracellular contents; inhibition of respiratory chain enzymes; and binding to phosphorus-containing compounds such as DNA, preventing cell reproduction [15,16,21–23]. The targets of action of SNs have been relatively well described in complex studies [21–23,25].

Recently, it was demonstrated that SNs affected the extracellular matrix composition and structure of *Candida* biofilms [26] and exhibited synergistic antibiofilm activity when combined with either nystatin or chlorhexidine digluconate [27]. In light of the therapeutic potential of SNs, one essential aspect that remains to be established is the impact of the chemical stability of SNs on their efficacy against *Candida* biofilms. Variables such as temperature, ionic strength, and nature of the immersion medium (composition and pH) may influence the size, rate of dissolution, aggregation, and stability of SNs [28–30]. The loss of chemical stability could cause aggregation of SNs and reduction of their effectiveness against biofilms. Thus, our aim in this study was to evaluate how the chemical stability of silver nanoparticles (SNs) influences their efficacy against *Candida albicans* and *C. glabrata* biofilms.

Materials and methods

Synthesis and characterization of silver colloidal nanoparticles

SNs were synthesized in accordance with a previously described procedure [28]. Briefly, 5.0×10^{-3} mol/l silver nitrate (Merck KGaA, Darmstadt, Germany) was reduced by the addition of 0.3 mol/l sodium citrate (Merck KGaA). The solution turned amber yellow and a colloidal suspension was formed. Then, the silver colloids were stabilized by the addition of 1.4 mol/l ammonia (Merck KGaA). SN suspensions were characterized by ultraviolet/visible (UV/vis) spectroscopy (Shimadzu MultSpec-1501 spectrophotometer; Shimadzu Corporation, Tokyo, Japan) and by transmission electron microscopy (FEG-VP Supra 35 electron microscope; Carl Zeiss, Jena, Germany). The typical absorption spectrum of SNs, centered at 430 nm, was located in the UV/vis spectrum; electron micrograph images displayed spherical SNs with an average particle size of 5 nm [14,31]. The SN concentration of 54 mg/l used in this study was based on its efficacy against *Candida* biofilms reported in previous studies [14,26,31].

Chemical stability of SNs through temperature and pH variations

Chemical stability assays were carried out at room temperature based on the method of Ellis et al. [32], with some modifications. For the temperature stability assay, stock suspensions of SNs in glass tubes were placed in a water bath at 50°C (SN-50), 70°C (SN-70), and 100°C (SN-100) for 30 min. The resultant SN colloidal suspensions were also characterized by UV/vis spectroscopy as cited above. Later, SN suspensions (preheated) were diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) to achieve a silver concentration of 54 mg/l.

For the pH stability assay, the pH of the SN suspensions at 54 mg/l (diluted in RPMI 1640 medium) was adjusted to pH 5.0 (SN-pH 5) or pH 9.0 (SN-pH 9) using solutions of 1 M hydrochloric acid and 1 M sodium hydroxide, respectively. After 2 h at room temperature, the pH was readjusted to pH 7.0.

Substrate preparation

The substrate used for biofilm formation was denture acrylic resin. For standardization of acrylic resin specimens, a stainless steel matrix with internal molds was fixed with wax (Wilson, São Paulo, Brazil) on a glass plate with a rough surface (to mimic the roughness of the palate)

and invested in metallic flask with type III dental stone (Herodent, Petrópolis, Brazil). After the dental stone set, the flask halves were separated, the wax was removed, and the matrix molds and glass plate were cleaned. The denture resin (QC20; Dentsply Ind. e Com. Ltd., Petrópolis, Brazil) was manipulated, packed, pressed into the matrix molds, and polymerized according to the manufacturer's recommendations. After bench cooling at room temperature, the specimens (10 × 10 × 3 mm) were deflasked, and the excess resin was removed with a bur (Maxi-Cut; Maillefer SA, Ballaigues, Switzerland). The acrylic specimens were then rinsed with deionized water, dried at room temperature, and sterilized [5].

Artificial saliva medium

Artificial saliva (AS; pH 6.8) was the medium used for biofilm formation [33]. Its composition was as follows: 2 g of yeast extract (Sigma-Aldrich), 5 g of peptone (Sigma-Aldrich), 2 g of glucose (Synth, Diadema, Brazil), 1 g of mucin (Sigma-Aldrich), 0.35 g of sodium chloride (Merck KGaA), 0.2 g of calcium chloride (Sigma-Aldrich), and 0.2 g of potassium chloride (Merck KGaA) per 1 l of deionized water.

Yeast strains and growth conditions

Two strains of *C. albicans* and *C. glabrata* were tested. In addition to the reference *C. albicans* (American Type Culture Collection [ATCC] 10231) and *C. glabrata* (ATCC 90030), two *Candida* oral clinical isolates were used, namely, *C. albicans* 324LA/94 (obtained from the culture collection of Cardiff Dental School, Cardiff, UK) and *C. glabrata* D1 (obtained from the Biofilm Group of the Centre of Biological Engineering, University of Minho, Braga, Portugal).

All yeast strains were grown aerobically on Sabouraud glucose agar medium (SGA; Difco, Le Pont de Claix, France) at 37°C for 24 h. A loopful of the *Candida* colonies from SGA plates was inoculated into Sabouraud glucose broth (SGB; Difco) medium and incubated at 37°C for 20–24 h under agitation (120 rpm). After being harvested by centrifugation (6500 *g* for 5 min at 15°C), cell pellets were washed twice in phosphate-buffered saline (PBS; pH 7, 0.1 M) and the cellular density adjusted to 1×10^7 cells/ml in AS, using a Neubauer hemocytometer. These *Candida* cell suspensions were used in the subsequent biofilm formation assays.

Biofilm formation and treatment with SNs

Candida biofilms were formed on acrylic resin specimens according to Silva et al. [5]. Briefly, acrylic specimens were placed in 24-well microtiter plates (Costar, Tewksbury, USA) and 1 ml of the standardized *Candida* cell suspension (1×10^7 cells/ml in AS) was added to each well and incubated at 37°C for 48 h under agitation (120 rpm). After 24 h, 500 μl of AS was removed and an equal volume of fresh AS was added. Following *Candida* biofilm formation (48 h), AS was removed and the acrylic specimens were washed once with 1 ml of PBS to remove nonadherent cells. Then, 1 ml of 54 mg/l SNs (with temperature and pH variations, as previously described) diluted in RPMI 1640 medium was added to the preformed *Candida* biofilms and incubated at 37°C for 24 h under agitation (120 rpm). For positive and negative controls, *Candida* biofilms were preformed on acrylic specimens and then incubated with 54 mg/l of SNs without temperature and pH variations and with RPMI 1640 medium without SNs, respectively.

To evaluate the influence of the length of treatment on the susceptibility of *Candida* biofilms to SNs, *Candida* biofilms (48 h) formed on acrylic specimens were incubated at 37°C (120 rpm) with 1 ml of RPMI 1640 medium containing SNs diluted at 54 mg/l (without temperature and pH variations) for 5 h and 24 h. These treatment periods were the same as those used by Harrison et al. [34] to examine the ability of *Candida* biofilms to survive exposure to various toxic metal ions. After each treatment period (5 h and 24 h), acrylic specimens were washed once with 1 ml of PBS to remove nonadherent cells. Controls devoid of SNs were also included.

Quantification of total biofilm biomass

Total biomass of *Candida* biofilms exposed to SNs (with or without temperature and pH variation) was measured using the crystal violet (CV) staining method [5,31]. Briefly, after the treatment period, acrylic specimens were washed once with 1 ml of PBS to remove loosely attached cells. Then, *Candida* biofilms were fixed with 1 ml of 99% methanol (Sigma-Aldrich). After 15 min, methanol was removed and the acrylic specimens were allowed to dry at room temperature. Next, 1 ml of CV stain (1% v/v; Merck KGaA) was added to each well containing acrylic specimens and incubated for 5 min. Acrylic specimens were gently washed with deionized water, dried at room temperature, and transferred to new 24-well microtiter plates. Then, 1 ml of acetic acid (33% v/v; Sigma-Aldrich) was added to remove the CV stain from the biofilms. Last, absorbance of the obtained solution was measured in a microtiter plate reader (Eon microplate spectrophotometer; BioTek, Winooski, VT, USA) at 570 nm and standardized in relation to the area of acrylic specimens (Abs/cm^2). The assays were performed independently three times in triplicate.

Quantification of biofilm viable cells

Cultivable *Candida* cells from biofilms exposed to SNs (with or without temperature and pH variation) were enumerated by counting colony-forming units (CFUs). Acrylic specimens were washed once with PBS, immersed in 1 ml of PBS in falcon tubes, sonicated for 30 s at 40 W, and vortexed for 5 min. Serial decimal dilutions (in PBS) of each biofilm cell suspension were plated on SGA, and the plates were incubated at 37°C. After 24 h, the total number of colony-forming units per unit area (Log_{10} CFU/cm^2) of acrylic specimens was enumerated. The experiments were performed independently three times in triplicate.

Statistical analysis

The data for each test were analyzed statistically using one-way analysis of variance and the Bonferroni post hoc test, using SPSS software (Statistical Package for the Social Sciences, Inc., Chicago, IL, USA) with significance level denoted at $P < 0.05$.

Results

The absorption spectra for the nonheated SNs (Fig. 1A), SN-50 (Fig. 1B), and SN-70 (Fig. 1C) were similar and demonstrated plasmon bands centered at approximately 430 nm. For SN-100, the absorption peak was attained at approximately 500 nm (Fig. 1D).

With reference to biofilm biomass quantification, Figure 2A shows that preheating of the SN suspension to 100°C impaired its efficacy only for *C. albicans* 324LA/94. In this case, SN-100 produced a significant increase (42.9%; $P = 0.037$) in the total biomass compared with the positive control group. The results displayed in Figure 3A indicate that for both strains of *C. glabrata*, the variation in pH significantly impaired the efficacy of SNs in reducing total biomass. When the SN-pH 5 group was compared with the positive control, increases in the total biomass of *C. glabrata* ATCC 90030 and *C. glabrata* D1 of 42.9% ($P = 0.036$) and 25% ($P = 0.045$), respectively, were observed. In addition, when the pH was changed to 9.0, the total biomass increased approximately 38% ($P = 0.036$) for *C. glabrata* ATCC 90030 and 39% ($P = 0.009$) for *C. glabrata* D1 compared with the positive control.

As shown in Figure 4A, *C. albicans* ATCC 10231, *C. albicans* 324LA/94, *C. glabrata* ATCC 90030, and *C. glabrata* D1 biofilms treated with SNs for 5 h showed significant reductions in total biomass of 47.2% ($P < 0.001$), 35.8% ($P < 0.001$), 51.5% ($P < 0.001$), and 36.8% ($P < 0.001$), respectively, compared with their respective controls (5 h without SN). When these biofilms were treated for 24 h, the reductions in the total biomass compared with the control groups were also significant for all strains as follows: *C. albicans* ATCC 10231: 18.5%, $P = 0.011$; *C. albicans* 324LA/94: 32.3%, $P < 0.001$; *C. glabrata* ATCC 90030: 22.3%, $P = 0.001$; and *C. glabrata* D1: 17.6%, $P = 0.027$. Although the 5-h treatment resulted in a higher reduction in total biomass than the 24-h treatment, the difference between these treatments was only significant for *C. glabrata* ATCC 90030 ($P = 0.001$).

In terms of biofilm cultivable cell quantification, Figures 2B and 3B clearly show that the temperature and pH variations neither impaired nor improved significantly the efficacy of SNs against *Candida* biofilms. On the other hand, the treatments with SNs for 5 h and 24 h (Fig. 4B) produced significant decreases in the number of colony-forming units only for *C. albicans* 324LA/94 (reduction of 1.44-log_{10}; $P < 0.001$) and *C. glabrata* D1 (reduction of 0.71-log_{10}; $P = 0.034$), respectively, compared with the controls. The comparison between the two treatment periods was statistically significant only for *C. albicans* 324LA/94 ($P < 0.001$), with better results for the shorter treatment period (5 h).

Discussion

There are few reports on how the chemical stability of SNs influences antimicrobial properties. Here, our goal was to determine whether heating or changing the pH of a SN stock solution and whether varying the treatment period would affect the antifungal activity of SNs against *Candida* biofilms. Clinically, SNs could be used in mouthwashes and as a disinfectant solution for soaking dentures. Therefore, in the present study, these nanoparticles were heated to 50°C, 70°C, and 100°C. These higher temperatures were used because storage temperatures for colloidal suspensions in some tropical countries and desert regions can exceed 50°C. Moreover, 100°C was chosen to represent an extreme condition and was close to the temperature used to synthesize SNs. Preheating SNs to 50°C, 70°C, and 100°C had no significant influence on total biomass or cell viability of *Candida* biofilms (Fig. 2). The only exception was the *C. albicans* 324LA/94 biomass, where SNs heated to 100°C induced a 42.9% increase in total biomass in relation to the no-heated group (positive control). In an attempt to explain these findings, additional tests were performed to verify the behavior of the SN suspension when subjected to heating.

Aliquots of each SN suspension heated to 50°C, 70°C, and 100°C were evaluated using UV/vis spectroscopy (Fig. 1). Surprisingly, SN-50 and SN-70 suspensions did not show extreme changes in their spectral absorption aspects when compared with the nonheated SN suspension, indicating that these suspensions remained stable after 30 min of heating. This is in agreement with results for total biomass

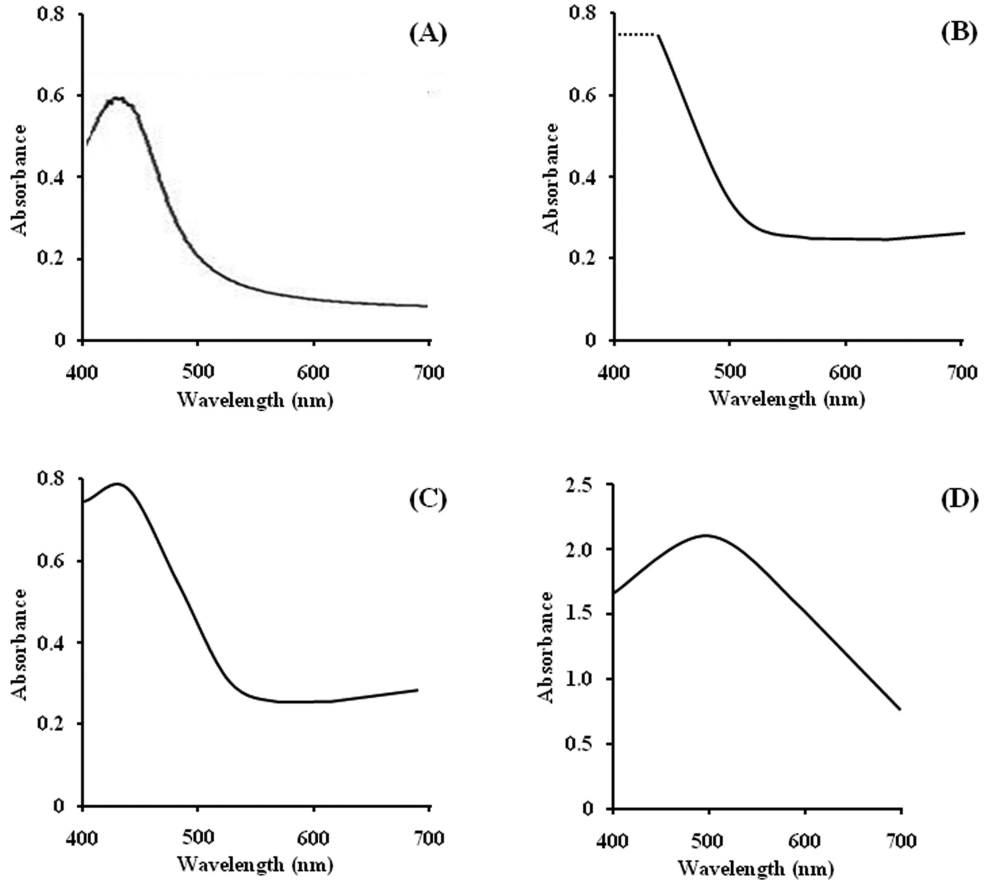

Figure 1. Ultraviolet/visible spectra of colloidal suspensions of silver nanoparticles not heated (A) and heated to 50°C (B), 70°C (C), and 100°C (D).

and cultivable biofilm cells obtained for SN-50, SN-70, and the control (SN without heating) and explains why there were no significant differences among these groups.

Kittler et al. [29] evaluated the dissolution in water of SNs stabilized with citrate and polyvinylpyrrolidone for up to 125 days at 5°C, 25°C, and 37°C. They found that SNs dissolved partially into ions and that an increase in temperature caused an increase in the degree of dissolution and release of SIs. As a result of this process, a considerable increase in the toxicity of SNs was observed. In the present study, although the nanoparticles were heated to high temperatures, the short heating period (30 min) may have been insufficient to a significantly dissolve these particles and release large amounts of SIs, in turn, improving antibiofilm activity. It must be emphasized that the SNs used in our study were well stabilized with ammonia. Ammonia's role is to trap all SIs present in the colloidal suspension by forming soluble diamine silver complexes; these complexes prevent the growth of already-formed SNs as well as the formation of new nanoparticles [28].

However, heating the SN suspension to 100°C changed the suspension's color and the particle size distribution. This is shown in Figure 1D by the shift of the absorption peak maximum towards a higher wavelength and the peak broadening of the UV-vis spectra. This might indicate that the SN-100 suspension was significantly destabilized, leading to the formation of silver aggregates or new nanoparticles [35]. However, the efficacy of the SN-100 suspension against *Candida* biofilms was significantly reduced only for the *C. albicans* 324LA/94 biomass. Unfortunately, there is no feasible explanation for this finding. These results point to the fact that the storage of colloidal suspension at high temperatures may not compromise the effectiveness of SNs against *Candida* biofilms. Perhaps, this is due to the inherent tendency for SN agglomeration when in contact with biofilms [26,36].

Regarding the tests performed to evaluate the efficacy of SN suspensions with a change in pH, it is important to note that pH 5.0 was chosen because it is close to the average pH of saliva from patients with denture stomatitis [37]; pH 9.0 was arbitrarily chosen as representative of a basic pH. After adjusting the pH (to 5.0 or 9.0) of SN colloidal suspensions diluted in RPMI 1640 medium, dark aggregates or flocs settled on the vessel bottom, indicating instability of the colloidal suspensions at both acidic and basic pH. For this reason, UV/vis spectroscopy analysis was not performed.

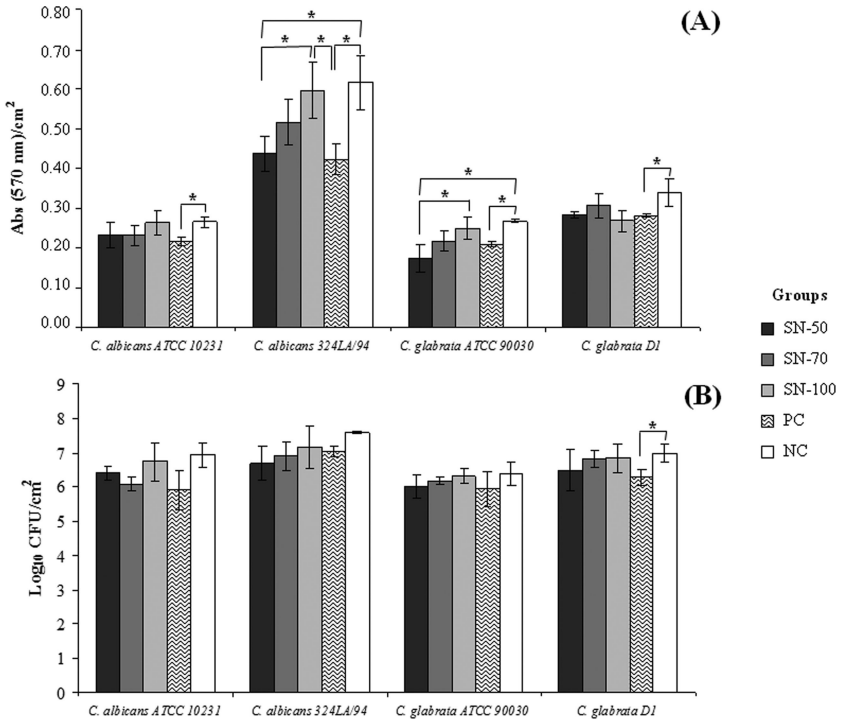

Figure 2. (A) Mean values of the absorbance per square centimeter obtained with crystal violet staining assay and (B) means of the logarithm of colony-forming units per square centimeter for mature *Candida* biofilms (48 h) after 24 h of treatment with silver nanoparticles (SNs) at 54 mg/L that were preheated to 50°C (SN-50), 70°C (SN-70), and 100°C (SN-100). Positive control (PC), biofilms incubated with unheated SNs. Negative control (NC), biofilms incubated with Roswell Park Memorial Institute 1640 medium without SNs. Error bars represent standard deviation of the mean. Asterisk (*) denotes $P < 0.05$, using analysis of variance followed by the Bonferroni test.

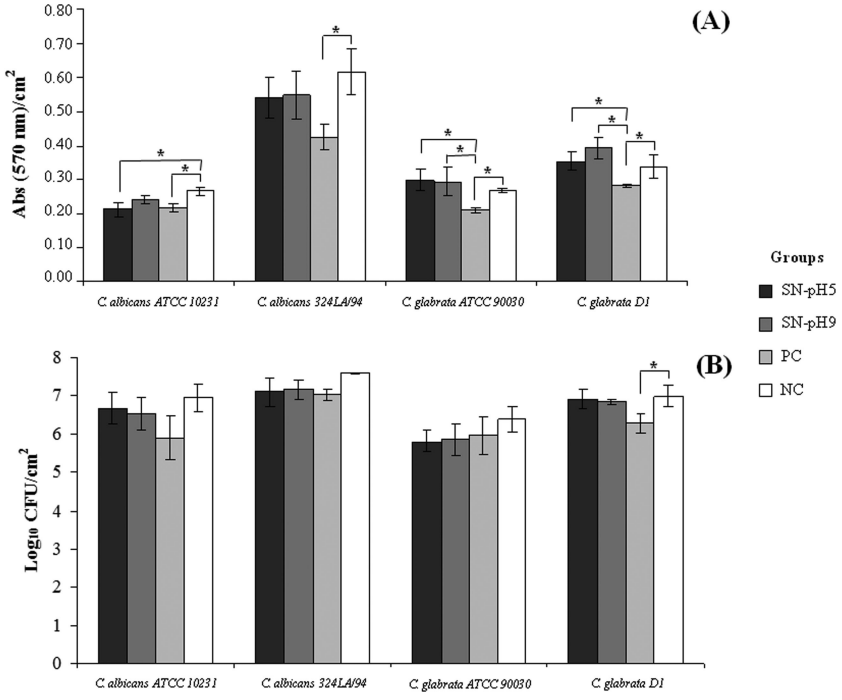

Figure 3. (A) Mean values of the absorbance per square centimeter obtained with crystal violet staining assay and (B) means of the logarithm of colony-forming units per square centimeter for mature *Candida* biofilms (48 h) after 24 h of treatment with silver nanoparticles (SNs) at 54 mg/L that had the pH varied (during 2 h) to 5.0 (SN-pH 5) and 9.0 (SN-pH 9) before contact with biofilms. Positive control (PC), biofilms incubated with SNs without pH variation. Negative control (NC), biofilms incubated with Roswell Park Memorial Institute 1640 medium without SNs. Error bars represent standard deviation of the mean. Asterisk (*) denotes $P < 0.05$, using analysis of variance followed by the Bonferroni test.

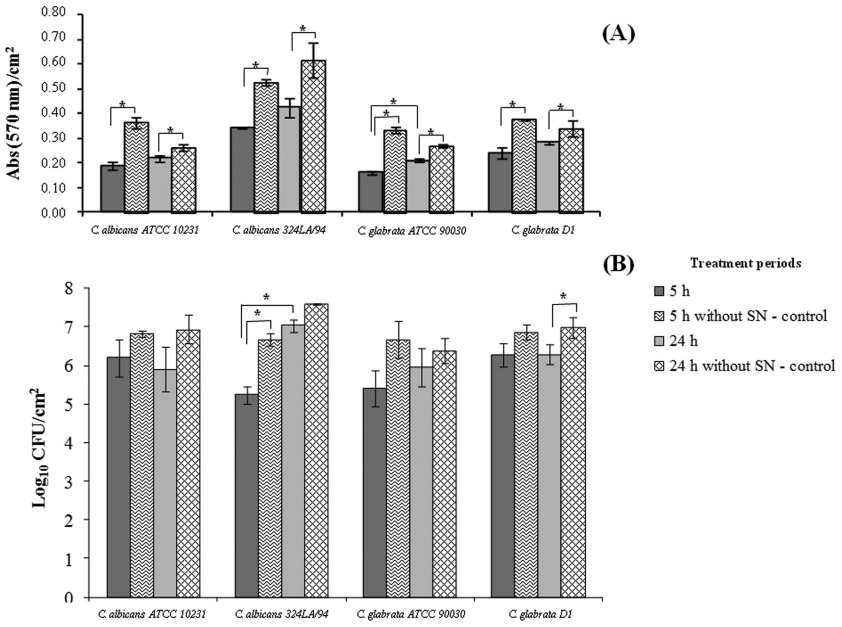

Figure 4. (A) Absorbance values per square centimeter obtained with crystal violet staining assay and (B) means of the logarithm of colony-forming units per square centimeter for mature *Candida* biofilms (48 h) after different treatment periods with silver nanoparticles at 54 mg/L. Error bars indicate standard deviation of the mean. Asterisk (*) denotes $P < 0.05$, using analysis of variance followed by the Bonferroni test.

According to Kittler et al. [29], the dissolution behavior of nanoparticles in biological medium is definitely more complex and distinct from that in water due to the presence of several organic compounds in the medium. RPMI 1640 medium contains substantial amounts of glucose, amino acids, and proteins. Thus, the addition of hydrochloric acid or sodium hydroxide probably led to a destabilization in the colloidal suspension and favored the release of SIs, which may have bound to proteins or precipitated in the RPMI medium as silver chloride [38], silver phosphate [29], or silver hydroxide.

The results also demonstrate that the variation in pH did not impair or improve significantly the efficacy of SNs on the number of colony-forming units (Fig. 3B). The reasons for this are related to those mentioned above regarding the effect of temperature stability on the antifungal activity of SNs. However, the pH changes resulted in significant increases in the total biomass of *C. glabrata* ATCC 90030 and *C. glabrata* D1 when compared with the positive controls (Fig. 3A). As *C. albicans* biofilms are more profuse and produce greater amounts of extracellular matrix than *C. glabrata* biofilms [7,39], the nanoparticles and some aggregates were likely retained in the matrix and did not reach the cells that were in the deeper layers of the biofilm. If this is the case, it is possible that the contact of aggregates with their cells may have been facilitated in *C. glabrata* ATCC 90030 and *C. glabrata* D1 biofilms. Although it did not result in a decrease in the number of viable cells (probably due to SN aggregation), it might have generated a stress response in the biofilm cells; the cells responded by producing more matrix, since the number of viable cells was similar. The lack of correlation between both methods used for biofilm quantification was not surprising, given that the two methods assessed different biofilm features and are accepted as complementary. While CV staining quantified the total biofilm biomass (extracellular matrix and both living and dead cells), the CFU enumeration determined the number of cultivable cells.

Additionally, by comparing the treatment periods (5 h and 24 h), there were differences for the biomass of *C. glabrata* ATCC 90030 biofilm and for the number of colony-forming units of *C. albicans* 324LA/94 biofilm, with better results for the shorter treatment period for all (Fig. 4). It is likely that the exposure period (5 h) was too short to cause a large aggregation of SNs, in turn, facilitating their antimicrobial action. From a clinical perspective, these results are important because shorter exposures to SNs could prevent the harm that is related to silver toxicity for mammalian cells. What should be considered are the inherent physiological differences among the species and strains tested [39]. For instance, biofilms formed by *C. albicans* normally have a thin basal yeast layer and a thicker hyphal layer (less compact), whereas *C. glabrata* biofilms are characterized by a compact monolayer or multilayer of yeasts [39]. These features may hinder or facilitate the action of SN against *C. albicans* and *C. glabrata* biofilms.

Based on the results of our study, the use of stabilized or destabilized SNs may not be crucial to how SNs affect

against *Candida* biofilms. Additional research to elucidate the longevity of SN formulation, especially under oral temperature and pH conditions, is needed. In addition, research on the toxicological effects that result when nanoparticles are changed by environmental variations is needed. Moreover, studies on the incorporation of SNs into acrylic resin and on the physical, mechanical, and microbiological properties of the nanocomposites generated are also necessary in order to develop biomaterials that will prevent or control *Candida*-associated denture stomatitis.

In summary, the results indicate that temperature and pH variations of SNs did not affect their efficacy against the sessile cells of *Candida* biofilms. Furthermore, the treatment periods were not decisive in terms of the susceptibility to SNs, and the shorter treatment period might be clinically advantageous. Accordingly, these results provide important insights that could be useful in the treatment of *Candida*-associated denture stomatitis with formulations and biomaterials containing SNs.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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