JOURNAL OF PROSTHODONTIC RESEARCH 59 (2015) 42–48

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Original article

Susceptibility of *Candida albicans* and *Candida glabrata* biofilms to silver nanoparticles in intermediate and mature development phases

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ARTICLE INFO

Article history:
Received 21 January 2014
Received in revised form
26 June 2014
Accepted 30 July 2014
Available online 26 August 2014

Keywords:
Silver nanoparticles
Denture stomatitis
Biofilms
Candida albicans
Candida glabrata

ABSTRACT

Purpose: The aim of this study was to investigate the susceptibility of *Candida albicans* and *Candida glabrata* biofilm development, in their intermediate and maturation stages, to the influence of silver nanoparticles (SN).

Methods: SN (5 nm) suspensions were synthesized *via* reduction of silver nitrate by a solution of sodium citrate. These suspensions were used to treat *Candida* biofilms for five hours, grown on acrylic surfaces for 24-h (intermediate stage) and 48-h (maturation stage), and their efficacy was determined by total biomass (using crystal violet staining) and colony forming units (CFUs) quantification.

Results: SN promoted significant reductions ($p < 0.05$) in the total biomass and number of CFUs of *Candida* biofilms, ranging from 23% to 51.5% and 0.63 to 1.59-$\log_{10}$, respectively. Moreover, there were no significant differences in the total biofilm biomass ($p > 0.05$), when the different stages of biofilm development (24 or 48 h) were exposed to SN. Comparing the number of CFUs between 24- and 48-h biofilms treated with SN, a significant difference ($p < 0.05$) was found only for the *C. albicans* 324LA/94 strain.

Conclusions: In general, the intermediate and maturation stages of biofilm development do not interfere in the susceptibility of *C. albicans* and *C. glabrata* biofilms to SN. These findings are fundamental for the deployment of new therapies aimed at preventing denture stomatitis.

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http://dx.doi.org/10.1016/j.jpor.2014.07.004
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1. Introduction

Candida-associated denture stomatitis is a form of oral *Candida* infection, covering areas of the mucosa which support the upper denture [1]. This infection is prevalent among individuals under continuous use of drugs, with suppressed immune systems, nutritional deficiencies and precarious denture hygiene [2,3]. Moreover, it is associated with biofilm formation by *Candida* species, of which *Candida albicans* and *Candida glabrata* are the most abundant in the oral cavity [4].

The dynamic of *Candida* biofilm development on polymethylmethacrylate basically occurs in three stages: (i) early (0–11 h), characterized by adhesion of blastospores to the surface and formation of microcolonies [5]; (ii) intermediate (12–30 h), represented by the proliferation of fungal cells (due to cell budding) and their deposition on areas of irregularities, and by the emergence of extracellular matrix production [5], and (iii) maturation (31–72 h), in which the matrix production increases with incubation time and the fungal colonies become covered by this matrix, forming structures with complex architectures [5]. An *in vivo C. albicans* biofilm denture model (using rodents) has been developed to mimic human denture stomatitis [6]. Not surprisingly, observation of *Candida* biofilm formation using this *in vivo* model was comparable to that noted in *in vitro* [5], showing the following stages of development: 6 h, yeast cells adhered on the acrylic surface; 24 h, a confluent layer of cells covering the surface and the start of extracellular matrix production; 48 h, a mature biofilm comprising yeast, hyphae and host cells surrounded by the extracellular matrix [6]. Biofilms are associated with antimicrobial resistance [7] and, therefore, infections related to them are recalcitrant and tricky to treat [8].

Several studies [5,9,10] showed age-related differences of biofilms in response to antimicrobial agents. Chandra et al. [5] found that the minimum inhibitory concentrations (MICs) of amphotericin B, nystatin, fluconazole, and chlorhexidine were significantly higher for intermediate and mature *C. albicans* biofilms than for earlier biofilms. Another study revealed that mature bacteria biofilms were more resistant to chlorhexidine preparations than young counterparts [10]. The presence of an extracellular matrix probably hinders the penetration of drugs in mature biofilms, and contributes to their resistance [11].

This fact has stimulated the use of nanotechnology to generate new antifungal strategies to control *Candida* biofilm formation in denture stomatitis. Currently, silver nanoparticles (SN) have been tested against biofilms of *Candida* species [12–17]. These nanoparticles, at concentrations ranging from 54 to 216 μg/mL, promoted significant reductions in the total biomass and in the number of cultivable *Candida* biofilm cells [12,14,16]. Previous results showed that SN combined with either nystatin or chlorhexidine digluconate exhibited synergistic antibiofilm activity dependent on the *Candida* species and the drug concentrations used [17]. In addition, the nanoparticle size and the type of stabilizing agent in the silver colloidal nanoparticles did not interfere in their antifungal activity against *C. albicans* and *C. glabrata* biofilms [14]. The targets of antimicrobial action of SN are well described in interesting studies [18–21].

Although the literature displays encouraging findings about the effect of SN against *Candida* biofilms at early stage [12], an important point needs to be investigated: the susceptibility of *Candida* biofilms at intermediate and maturation stages of development to SN. The evaluation of the physiological stages of biofilm development on the susceptibility of *Candida* biofilms to SN is important for the knowledge of their antibiofilm spectrum of action. Thus, the findings of this study may contribute to broaden or restrict the use of SN in future clinical applications, such as its direct incorporation into acrylic resin or other polymers, adhesives, or varnishes, and its use as a decontamination solution of complete dentures. Therefore, the aim of this study was to investigate the susceptibility of *C. albicans* and *C. glabrata* biofilm development, in their intermediate and maturation stages, to the influence of SN, through quantification of total biomass and cultivable cells. The null hypothesis was that those two stages of biofilm development would not differ in the susceptibility to SN.

2. Materials and methods

2.1. *Synthesis of silver colloidal nanoparticles*

Silver nanoparticles (SN) were synthesized *via* reduction of silver nitrate ($AgNO_3$ – Merck KGaA, Darmstadt, Germany) at 5.0×10^{-3} mol/L by a solution of sodium citrate ($Na_3C_6H_5O_7$ – Merck KGaA) at 0.3 mol/L [22]. A colloidal suspension was formed when the solution turned yellow. Next, SN were stabilized by adding a solution of ammonia (NH_3 – Merck KGaA) at 1.4 mol/L. These nanoparticles were characterized as described previously [12], and the average size of SN obtained was 5 nm.

2.2. *Preparation of acrylic resin specimens*

Acrylic resin specimens were used as substrates for biofilm development. A stainless steel matrix with internal molds was fixed with wax (Wilson, São Paulo, Brazil) on a glass plate with rough surface (to mimic the palatal roughness) and invested with type III dental stone (Herodent, Petrópolis, Brazil) into a denture flask. After the dental stone had set, the flask was opened and the matrix molds and the glass plate were cleansed with acetone. Powder and liquid denture resin (QC20, Dentsply Ind. e Com. Ltd., Petrópolis, Brazil) were proportioned, mixed, and pressed into the matrix molds and polymerized according to the manufacturer's instructions. The acrylic specimens (10 mm $\times$ 10 mm $\times$ 3 mm) were bench-cooled overnight before deflasking. Then, the excess resin was removed with a bur (Maxi-Cut; Maillefer SA, Ballaigues, Switzerland). The specimens were rinsed three times with deionized water, dried at room temperature, packaged in aluminum foil and autoclaved at 121 °C for 15 min [15].

2.3. *Candida strains and culture conditions*

Two reference strains from American Type Culture Collection (ATCC), *C. albicans* (ATCC 10231) and *C. glabrata* (ATCC 90030),

were used in this study. Moreover, two *Candida* oral clinical isolates were also examined, 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 strains were subcultured on Sabouraud dextrose agar medium (SDA, Difco, Le Pont de Claix, France) at 37 °C for 24 h. Then, yeast cells were inoculated in Sabouraud dextrose broth (SDB; Difco) medium and incubated at 37 °C for 20–24 h under agitation (120 rpm). Yeast cells were harvested by centrifugation after the incubation period (6500 × *g*, for 5 min at 15 °C), washed twice in phosphate buffered saline (PBS; pH 7, 0.1 M) and the cellular concentration was adjusted to 1×10^7 cells/mL in artificial saliva (AS) medium [12], using an improved Neubauer chamber.

2.4. Biofilm development and treatment with SN

Candida biofilms were developed on acrylic resin specimens in 24-well microtiter plates (Costar, Tewksbury, USA) containing 1 mL of each *Candida* cell suspension (1×10^7 cells/mL in AS). To form biofilms at intermediate stage (24-h biofilms), the plates were incubated at 37 °C for 24 h under agitation (120 rpm), while to generate biofilms at maturation stage (48-h biofilms), the plates were incubated for 48 h under the same conditions. For 24-h biofilms, after 12 h, 500 μL of AS were withdrawn and an equal volume of fresh AS was added, while for 48-h biofilms, the AS medium was renewed after 24 h. Following *Candida* biofilm development (24 and 48 h), the specimens were washed once with 1 mL of PBS to remove non-adherent cells. Next, 1 mL of 54 μg/mL [12,14,16] of SN diluted in RPMI 1640 medium (Sigma–Aldrich, St. Louis, USA) was added to the biofilm-containing wells. RPMI 1640 without SN was added to the wells designated for controls. The plates were then re-incubated under agitation in a shaker (120 rpm) at 37 °C for 5 h.

2.5. Total biofilm biomass assay

The total biomass of *Candida* biofilms, after treatment, was analyzed by using the crystal violet (CV) staining method [12,15]. The medium was aspirated and the acrylic specimens were gently washed with 1 mL of PBS to remove the planktonic cells. Fixation was performed by adding 1 mL of 99% methanol (Sigma–Aldrich) to the *Candida* biofilms and removing it after 15 min. Acrylic specimens were allowed to dry at room temperature. Subsequently, 1 mL of CV stain (1%, v/v) (Merck KGaA) was added into each well containing acrylic specimens and incubated for 5 min. The excess of crystal violet was withdrawn by washing the specimens with deionized water. Crystal violet bound to the biofilms was detached using 1 mL of acetic acid (33%, v/v) (Sigma–Aldrich). Finally, an aliquot (200 μL) of the obtained solution was transferred to a 96-well plate and the absorbance was then measured in a microtiter plate reader (Eon Microplate Spectrophotometer; Bio Tek, Winooski, USA) at 570 nm and standardized in relation to the area of acrylic specimens (Abs/cm^2). The assays were performed, independently and in triplicate, at least three times.

2.6. Quantification of biofilm cells assay

The quantification of *Candida* cultivable cells from biofilms treated with SN was carried out by counting colony-forming units (CFUs). For this, the acrylic specimens were washed with PBS, sonicated (30 s at 40 W) and vortexed (5 min) into falcon tubes containing 1 mL of PBS. Each biofilm cell suspension was serially diluted in PBS and plated on SDA. After incubation at 37 °C for 24 h, the total number of CFUs per unit area ($\log_{10}$ CFU/cm^2) of acrylic specimens was enumerated. The experiments were performed, independently and in triplicate, at least three times.

2.7. Statistical analysis

The normality of the data was verified using the Shapiro–Wilk test. Afterwards, parametric statistical analysis was performed for each test using two-way ANOVA, and *post hoc* Holm–Sidak test using SigmaPlot 12.0 software. Treatment with and without SN, and the biofilm stage (intermediate and maturation), were considered as variation factors. All tests were performed with a confidence level of 95%.

3. Results

In accordance with Fig. 1, it was observed that the 24-h biofilms of *C. albicans* ATCC 10231, *C. albicans* 324LA/94, *C. glabrata* ATCC 90030 and *C. glabrata* D1 treated during 5 h with SN at a concentration of 54 μg/mL showed significant reductions in the total biomass of 23 ($p = 0.002$), 22.9 ($p < 0.001$), 42.9 ($p < 0.001$) and 27.7% ($p < 0.001$), respectively, compared to their respective controls. For 48-h biofilms (Fig. 1), *C. albicans* ATCC 10231, *C. albicans* 324LA/94, *C. glabrata* ATCC 90030 and *C. glabrata* D1 showed significant reductions in the 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 to their respective controls. Although untreated 48-h biofilms (controls) of *C. albicans* ATCC 10231, *C. albicans* 324LA/94 and *C. glabrata* ATCC 90030 generated a significantly ($p < 0.05$) higher biomass than 24-h counterparts, comparisons between 24- and 48-h biofilms treated with SN did not show significant differences in reducing total biomass ($p > 0.05$), for all strains. Thus, the stages of biofilm development tested (intermediate and maturation) did not interfere with the susceptibility of *Candida* biofilms to SN.

Concerning the effect of SN on the biofilm cultivable cells (Fig. 2), 24-h biofilms of *C. albicans* ATCC 10231, *C. albicans* 324LA/94, *C. glabrata* ATCC 90030 and *C. glabrata* D1 treated for 5 h with SN showed decreases in the number of CFUs of, respectively, 0.27 ($p > 0.05$), 0.96 ($p = 0.002$), 1.59 ($p < 0.001$) and 1.03-$\log_{10}$ ($p = 0.005$), compared to the control groups. For 48-h biofilms (Fig. 2), the treatment with SN promoted a significant decrease in the number of CFUs for *C. albicans* ATCC 10231 (reduction of 0.63-$\log_{10}$, $p = 0.035$), *C. albicans* 324LA/94 (reduction of 1.44-$\log_{10}$, $p < 0.001$) and *C. glabrata* ATCC 90030 (reduction of 1.27-$\log_{10}$, $p = 0.005$), compared to the controls. Additionally, comparing the number of CFUs between 24- and 48-h biofilms treated with SN, significant differences were found ($p = 0.021$) only for *C. albicans* 324LA/94, with higher

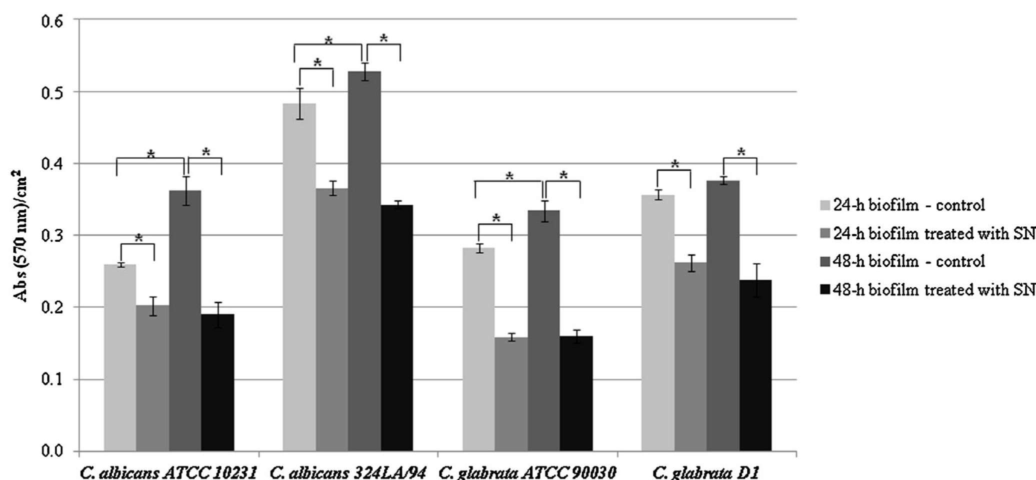

Fig. 1 – Absorbance values per cm^2 obtained with crystal violet staining assay for *Candida* biofilms at intermediate (24-h biofilm) and maturation (48-h biofilm) stages of development, after treatment with silver nanoparticles (SN) at 54 μg/mL during 5 h. Error bars indicate the standard deviations of the means. (*) Denote statistical difference between the groups by using two-way ANOVA and *post hoc* Holm–Sidak test with a confidence level of 95%.

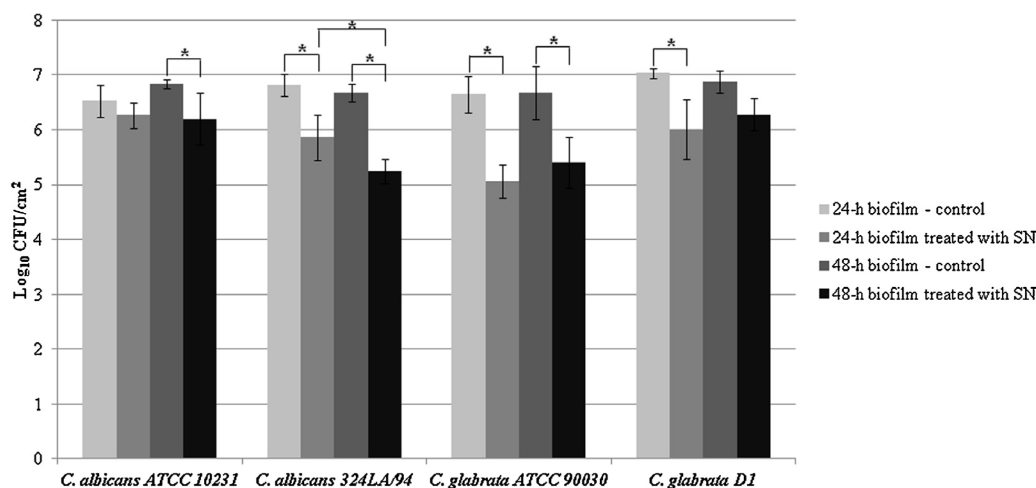

Fig. 2 – Logarithm of colony forming units per cm^2 obtained for *Candida* biofilms at intermediate (24-h biofilm) and maturation (48-h biofilm) stages of development, after treatment with silver nanoparticles (SN) at 54 μg/mL during 5 h. Error bars indicate the standard deviations of the means. (*) Denote statistical difference between the groups by using two-way ANOVA and *post hoc* Holm–Sidak test with a confidence level of 95%.

reduction in the number of CFUs for the mature biofilm (Fig. 2). For the other strains, these comparisons did not show significant differences ($p > 0.05$), and the stages of biofilm development tested did not reveal a significant influence on the susceptibility to SN.

4. Discussion

This study was carried out with 24-h and 48-h biofilms to encompass, respectively, the intermediate and the maturation stages of *Candida* species biofilms on acrylic strips. The effect of SN on *Candida* biofilms at early stage of development was demonstrated in a previous study [12]. Considering the data obtained in this study, it was possible to partially accept the null hypothesis since the two stages of *Candida* biofilm development only differed in susceptibility to SN for *C. albicans* 324LA/94 viable cells.

In general, mature biofilms are recalcitrant and more tolerant to antimicrobials. Some mechanisms have been proposed to explain their resistance, namely: decreased metabolic activity; altered gene expression; protective extracellular matrix, which may hamper the diffusion of drugs; and presence of persistent cells [23]. For instance, Anwar et al. [24] found that *Staphylococcus aureus* biofilms formed during a 4-day period exhibited higher decrease in viable cell counts when treated with tobramycin and cephalexin than 13-day-old biofilms. These authors used a variation between young and mature biofilms more marked (9 days) than that used in the present study (1 day). This

detail may clarify the fact that the data found in the two studies are distinct.

The intermediate stage of *Candida* biofilms is characterized by the emergence of extracellular matrix covering the fungal microcolonies, while in mature biofilms higher amounts of extracellular material and fungal cells completely involved by this material are evident [5,6]. Comparing the total biomass between 24- and 48-h untreated biofilms (controls), the results in Fig. 1 showed significant differences for *C. albicans* ATCC 10231, *C. albicans* 324LA/94 and *C. glabrata* ATCC 90030, with higher values for the mature biofilms. On the other hand, the proportions of cultivable cells (Fig. 2) in mature (48 h) untreated biofilms were similar to those of young counterparts (24 h). All these results indicate that the differences between 24- and 48-h biofilm biomasses occur exclusively due to increased production of extracellular matrix by 48-h untreated biofilms.

Interestingly, even with this increased matrix production by 48-h untreated biofilms, it was not possible to find differences in the susceptibility to SN between 24- and 48-h biofilms (Fig. 1). The treatment with SN probably produces a plateau of biomass accumulation regardless of the stage of biofilm development. Regarding cell viability (Fig. 2), the results showed the same trend, except for *C. albicans* 324LA/94. For this strain, curiously, 48-h biofilm was more susceptible to SN than 24-h biofilm. The reason for this phenomenon is unknown. However, it is believed that the observed pattern is due to the low variability of the data obtained for the 48-h treated biofilm compared to the variability of the data for the 24-h treated biofilm.

Similar results were found by Chandra et al. [5]. These authors determined the MICs of amphotericin B, nystatin, fluconazole and chlorhexidine for early, intermediate, and mature *C. albicans* biofilms. They observed that during early biofilm stage the MICs were 0.5, 1, 8, and 16 μg/mL for amphotericin B, fluconazole, nystatin and chlorhexidine, respectively. However, after 24 and 48 h (intermediate and maturation stages), the MIC values did not differ and reached values of 8, 128, 16, and 256 μg/mL for amphotericin B, fluconazole, nystatin and chlorhexidine, respectively.

The highlight of the present study's findings is that, in general, mature *C. albicans* and *C. glabrata* biofilms (48 h) were not more tolerant against SN than intermediate biofilms of these *Candida* species. This fact may indicate the use of SN to, both prevent biofilms to reaching more advanced stages of development, and combating preformed biofilms already in the stage of maturation. Consequently, the same SN concentration might have a broader clinical applicability at preventing denture stomatitis. We recommend further assays testing antifungals against different stages of biofilms, since it could have an impact on time and material consumption in research involving *Candida* biofilms. Thus, a stage of biofilm development of 24 h may serve as a standard for future *in vitro* susceptibility tests.

Our previous work showed that SN had a more pronounced effect against adhered cells (early stage of biofilm formation) of the same strains of *C. albicans* and *C. glabrata*, achieving reductions around 85–90% in the total biomass and 6.5-log_{10} in the number of CFUs [12]. The early stage is characterized by lower amounts of cells and in a metabolically excited state [25]. Taken together, it may have increased the efficacy of the SN against *Candida* species since their paths were ‘‘cleaner’’ to reach these targets.

Regarding the biofilm quantification results, it should be emphasized that CV staining assay does not allow differentiation between living and dead cells, so it was used as a complement trial to CFU enumeration. In general, significant reductions were observed in the total biomass and in the number of CFUs ranging from 23% to 51.5% (Fig. 1) and 0.63 to 1.59-log_{10} (Fig. 2), respectively. This highlights the effect of SN in the biofilm matrix and also in their cells. SN may have dissolved part of the extracellular matrix material, diffused inside the biofilm matrix through the pores and reached the cells in the deeper layers [26]. When in contact with the fungal cells, these nanoparticles preferably attack their membranes, causing disruption of membrane potential and subsequent cell death [19]. Moreover, SN may inhibit respiratory chain enzymes and interact with the DNA of microorganisms, preventing cell reproduction [18,20,21].

Furthermore, the silver concentration tested in the present study (54 μg/mL) can be considered low, when compared with the concentrations of conventional antifungal drugs used in some studies. Fonseca et al. [27] evaluated the fungicidal activity of fluconazole against 24-h *C. glabrata* biofilms and found that this agent, at concentrations ranging from 50 to 1250 μg/mL, was ineffective in reducing total biofilm biomass and number of CFUs. In the study of Tobudic et al. [28], posaconazole at concentrations of 2 and 256 μg/mL also was ineffective in reducing the number of CFUs of *C. albicans* biofilms. Vandenbosch et al. [29] verified that the treatment with miconazole at 2081 μg/mL resulted in a significant reduction (ranging from 89.3% to 99.1%) in the number of CFUs for *C. albicans*, *C. glabrata*, *Candida Krusei*, *Candida parapsilosis* and *Candida tropicalis* biofilms.

Although SN have been studied as a possible antifungal agent in order to prevent denture stomatitis, the good mechanical hygiene [30] and removal of dentures during nocturnal sleep should not be neglected for maintenance of a healthy mucosa. Our research group is working to assess the most advantageous formula of SN against *Candida* biofilms without damage to human cells. In the future, these nanoparticles might be incorporated in denture base resins, with safety for human health, or used as a solution for denture decontamination. Studies focused on preventing denture stomatitis are very important due to the increase in the elderly population and complete denture wearers, mainly in developing countries [31].

Finally, the understanding of the relationship between the physiological stages of biofilm development and the efficacy of SN may provide insights for the conception of new therapies targeted to control *Candida* infections. Further, results about the antibiofilm effect of SN are promising and should inspire *in situ* and *in vivo* investigations with formulations or materials based on SN to manage *Candida*-associated denture stomatitis.

5. Conclusions

Within the limitations of the present study, it was concluded that:

- The intermediate and maturation stages of biofilm development do not interfere in the susceptibility of *C. albicans* and *C. glabrata* biofilms to SN regarding total biomass and cultivable cells, except for *C. albicans* 324LA/94 CFU enumeration.
- The knowledge of the relationship between the stages of biofilm development and the effect of SN is crucial for the deployment of new therapies aimed at preventing and controlling of *Candida*-associated denture stomatitis.

Conflict of interest

The authors claim to have no financial and personal relationships with other people or organizations that could inappropriately influence this work.

Acknowledgments

This study was supported by the São Paulo Research Foundation (FAPESP), Brazil, process 2009/15146-5. The authors are indebted to LIEC-CMDMC and INCTMN/FAPESP-CNPq in the name of Andressa Kubo for preparing and characterizing the colloidal suspensions of silver nanoparticles. We also thank Dr. David Williams (Cardiff University, Cardiff, UK) for providing the strain 324LA/94, and George Duchow for the English review.

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