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Análise da ação de nanopartículas de prata sobre o biofilme de espécies de *Candida*

Tese apresentada à Faculdade de Odontologia do Campus de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho"- UNESP, para obtenção do Título de DOUTOR EM ODONTOLOGIA (Área de concentração em Prótese Dentária).

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Co-orientadora: Profª. Drª. Fernanda Lourenção Brighenti

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RESUMO GERAL

O objetivo deste estudo foi avaliar a atividade antifúngica de nanopartículas de prata (NP) contra os biofilmes de *Candida albicans* e *Candida glabrata*. NP foram sintetizadas por meio da redução do nitrato de prata com citrato de sódio e estabilizadas com amônia ou polivinilpirrolidona. Os testes de concentração inibitória mínima (CIM) das NP contra células de *Candida* foram baseados no método da microdiluição. NP foram aplicadas sobre os biofilmes de *Candida* (48 horas) e após 24 horas de contato sua atividade antifúngica foi determinada por meio da quantificação da biomassa total (coloração com violeta cristal (VC)) e por meio da enumeração das unidades formadoras de colônias (UFCs). Após o tratamento com NP, as matrizes dos biofilmes foram extraídas e analisadas em termos de proteínas, carboidratos e DNA, e a estrutura dos biofilmes foi analisada por meio da microscopia eletrônica de varredura e de epifluorescência. A atividade antibiofilme da combinação de NP com nistatina e clorexidina foi avaliada por meio dos ensaios de VC e UFCs. Leveduras viáveis foram recuperadas a partir dos biofilmes previamente tratados com NP e adicionadas às células epiteliais HeLa e aos poços de placas de poliestireno e, após 2 horas de contato, a adesão foi determinada usando VC. A eficácia de NP submetidas às variações de temperatura (50, 70 e 100ºC) e pH (5 e 9) também foi avaliada, assim como a susceptibilidade às NP dos biofilmes de *Candida* em diferentes fases de crescimento. Os resultados de CIM mostraram que NP foram fungicidas contra os isolados testados em concentrações baixas (0,4-3,3 µg/mL). NP foram mais efetivas na redução da biomassa para os biofilmes de *C. glabrata* (reduções de 90% na concentração de 108 µg/mL) do que para *C. albicans*, e promoveram reduções significativas no $\log_{10}$ do número de UFCs em concentrações iguais ou superiores a 108 µg/mL. Os resultados demonstraram que o tipo de agente estabilizante e o tamanho das partículas não influenciaram na atividade antibiofilme. Por outro lado, as NP interferiram na composição da matriz dos biofilmes em termos de proteínas, carboidratos e DNA, e as imagens de

microscopia revelaram uma tendência de aglomeração destas partículas nos biofilmes e sugerem que as mesmas induziram danos nas paredes celulares das células. Quando as NP foram combinadas com nistatina e clorexidina observou-se atividade sinergística dependente das espécies e concentrações de drogas testadas. Ainda, a capacidade de adesão das leveduras viáveis às células epiteliais e poliestireno foi reduzida após tratamento prévio com NP, e esta redução foi maior quando os biofilmes foram tratados com NP na concentração de 54 µg/mL. Os resultados de estabilidade química mostraram que o pré-aquecimento a 100ºC e a variação do pH da suspensão coloidal afetaram adversamente a biomassa dos biofilmes de *C. albicans* e *C. glabrata*, respectivamente, não apresentando influência sobre o número de UFCs. Os períodos de formação do biofilme e de tratamento não foram determinantes sobre a susceptibilidade às NP. Todos esses achados podem auxiliar nas decisões terapêuticas com formulações contendo NP em pacientes com estomatite protética associada à *Candida*.

Palavras-chave: Biofilmes**,** *Candida*, Candidíase Bucal, Prata, Nanotecnologia.

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GENERAL ABSTRACT

The aim of this study was to evaluate the antifungal activity of silver nanoparticles (SN) against *Candida albicans* and *Candida glabrata* biofilms. Colloidal suspensions of SN were synthesized by reducing silver nitrate with sodium citrate and stabilized with ammonia or polyvinylpyrrolidone. Minimal inhibitory concentrations (MIC) were performed for *Candida* cells grown in suspension following the microbroth dilution method. *Candida* biofilms (48 h) were treated with SN for 24 h and then the total biomass quantification (by crystal violet (CV) staining) and the colony forming units (CFUs) were determined. Also, after treating with SN, extracellular matrices were extracted from *Candida* biofilms and analyzed chemically in terms of proteins, carbohydrates and DNA. To investigate the biofilm structure, scanning electron microscopy and epifluorescence microscopy were carried out. The antibiofilm activity of SN in combination with nystatin and chlorhexidine was also assessed by CV and CFU. Moreover, viable yeasts were recovered from the biofilms pretreated with SN and added to HeLa epithelial cells or to empty wells of polystyrene plates and, after 2 h of contact, the adhesion capacity of the yeasts was determined by using CV staining. The antibiofilm efficacy of SN subjected to variations of temperature (50, 70 and 100ºC) and pH (5 and 9) was also evaluated. Finally, the susceptibility to SN of biofilms in different stages of growth was analyzed. MIC results showed that SN were fungicidal against the tested strains at very low concentrations (0.4-3.3 µg/mL). SN were more effective in reducing the total biomass of *C. glabrata* (reductions around 90% at 108 µg/mL SN) than *C. albicans* biofilms, and provided significant $\log_{10}$ reduction of the number of CFUs after having being exposured to SN concentrations at or higher than 108 µg/mL. The results either demonstrated the particle size and the type of stabilizing agent used for producing SN did not interfere in their antifungal efficacy. On the other hand, SN interfered in the matrix composition of *Candida* biofilms in terms of protein, carbohydrate

and DNA, as well as the microscopy images revealed a trend of SN agglomeration within the biofilms and suggested that SN induced damage to cell walls of *Candida* isolates tested. When SN were combined with either nystatin or chlorhexidine it was observed synergistic antibiofilm activity, and this activity was dependent of the species and the drug concentrations. In addition, the adhesion capacity of viable yeasts to epithelial cells and polystyrene surface was reduced, and this reduction was higher when biofilms were pretreated with SN in a concentration of 54 µg/mL. Chemical stability results indicated that the preheating at 100ºC and the pH variation of the colloidal suspension only affected adversely the biomass of *C. albicans* and *C. glabrata* biofilms, respectively, with no significant influence on the number of CFUs. Additionally, the biofilm formation and treatment periods did not seem to be decisive on the susceptibility to SN. All these results may help guide therapeutic decisions with formulations containing SN in patients with *Candida*-associated denture stomatitis.

Key-words: Biofilms, *Candida*, Oral Candidiasis, Silver, Nanotechnology.

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INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

Biofilmes são comunidades microbianas estruturadas aderidas a uma superfície viva ou inerte em meio aquoso e envoltas por uma matriz de material exopolimérico (Costerton *et al*., 1999; Ramage *et al*., 2005). A sua formação ocorre como resultado de uma sequência de eventos, que incluem: união inicial reversível de células planctônicas, transição da união reversível para irreversível pela produção de polímeros extracelulares pelos microrganismos, desenvolvimento inicial da arquitetura do biofilme, seguida pelo desenvolvimento de microcolônias dentro do biofilme maduro (Van Houdt & Michiels, 2005). Ainda, durante este estágio, substâncias poliméricas extracelulares continuam a ser produzidas e arquiteturas complexas com canais de água e poros são formadas (Van Houdt & Michiels, 2005). O último estágio consiste na dispersão das células do biofilme para o ambiente circundante e o retorno para o estado planctônico (Van Houdt & Michiels, 2005).

Os biofilmes criam um ambiente que realça a resistência antimicrobiana (Lewis, 2001). As substâncias poliméricas extracelulares contêm consideráveis quantias de polissacarídeos, ácidos nucleicos, proteínas e lipídeos (Sutherland, 2001), os quais são responsáveis por manterem a integridade estrutural do biofilme e proporcionarem uma matriz ideal para o crescimento das células (Chaw *et al*., 2005). Ainda, as interações intercelulares servem para fortalecer a estabilidade mecânica das substâncias poliméricas extracelulares e a sobrevivência dos microrganismos (Chaw *et al*., 2005).

A formação do biofilme exerce um papel importante no desenvolvimento da estomatite protética, que afeta aproximadamente 50 a 70% dos indivíduos edêndulos portadores de próteses totais (Budtz-Jorgensen, 1990; Chandra *et al*., 2001). Este processo patológico acomete principalmente a mucosa palatina dos usuários de próteses totais (Jeganathan & Lin, 1992) e é caracterizado por um eritema difuso, ora homogêneo, ora representado por pontos ou áreas focais avermelhadas (Budtz-Jorgensen *et al*., 2000). Essas reações inflamatórias podem estar relacionadas a uma variedade de fatores relacionados à prótese, destacando-se: trauma por desadaptação protética, porosidades da base, uso por tempo prolongado e má higienização da prótese (Renner *et al*., 1979; Arendorf & Walker, 1987; Iacopino & Wathen, 1992; Como & Dismukes, 1994; Ramage *et al*., 2004; Salerno *et al*., 2011). Fatores sistêmicos como carências nutricionais, diabetes, hipertensão arterial, anemia, síndrome da imunodeficiência adquirida, quimioterapia antineoplásica e uso prolongado de medicamentos como antibióticos e corticosteroides podem aumentar a susceptibilidade para a instalação de candidose oral, considerando-se que a presença de algum fator predisponente diminua a resistência do hospedeiro (Samaranayake, 1990; Samaranayake *et al*., 2002). Ainda, alguns estudos mostram que, após *Candida albicans*, *Candida glabrata* é o microrganismo mais frequentemente associado com as candidoses orais (Li *et al*., 2007; Coco *et al*., 2008).

As candidoses associadas à estomatite protética não são de fácil tratamento e recidivas são frequentes após a interrupção do mesmo (Batista *et al*., 1999). A literatura mostra que os biofilmes de espécies de *Candida* são resistentes a diversos antifúngicos convencionais (Chandra *et al*., 2001) e os diferentes

mecanismos que podem estar envolvidos neste processo são: a) a alta densidade de células dentro dos biofilmes; b) os efeitos da matriz extracelular; c) a diminuição da atividade metabólica; 4) a expressão de genes resistentes e 5) a presença de células persistentes (Ramage *et al*., 2001; Jabra-Rizk *et al*., 2004; Kuhn & Ghannoum, 2004). Além disso, os fungos são organismos eucarióticos com estrutura e metabolismo similares àqueles dos hospedeiros eucariontes (Kim *et al*., 2009). Esses problemas representam um desafio na prática odontológica, em face de sua frequência, ao número de drogas antifúngicas disponíveis no mercado e à resistência aos antifúngicos adquirida por muitas espécies de *Candida* (Batista *et al*., 1999). Consequentemente, existe uma grande necessidade na descoberta de agentes antimicrobianos alternativos com novos mecanismos de ação.

Desde que Stoimenov *et al*. (2002) provaram que nanopartículas de óxidos de metais altamente reativos exibem excelente ação biocida contra bactérias gram-positivas e negativas, outras nanopartículas inorgânicas vêm sendo investigadas como agentes antimicrobianos. Nesse sentido, a prata tem sido incorporada em diversos materiais poliméricos na área da saúde (Bosetti *et al*., 2002; Rupp *et al*., 2004; Samuel & Guggenbichler, 2004; Li *et al*., 2006; Damm *et al*., 2008; Kong & Jang, 2008; Roe *et al*., 2008; Monteiro *et al*., 2012) por apresentar propriedades antimicrobianas para um largo espectro de microrganismos, incluindo bactérias gram-positivas e negativas (Sondi & Salopek-Sondi, 2004; Baker *et al*., 2005; Panácek *et al*., 2006; Pal *et al*., 2007) e fungos (Kim *et al*., 2008; Kim *et al*., 2009; Panácek *et al*., 2009).

Nanopartículas apresentam, além do menor tamanho das partículas, uma maior razão da área de superfície por volume e maior área disponível para oxidação e interação com os microrganismos do que as micropartículas (Monteiro *et al*., 2009). Ainda, tem sido relatado que o modo de ação antimicrobiana das nanopartículas de prata seja similar ao dos íons prata (Dibrov *et al*., 2002) e pode ser explicado da seguinte maneira: (1) interagem com proteínas contendo grupos sulfúricos ou grupos tiol nas membranas dos microrganismos, causando desregulação de sua permeabilidade e, com isso, a morte da célula microbiana, (2) ligam-se ao DNA, levando à desnaturação desta molécula e prevenção da reprodução celular e (3) bloqueiam a cadeia respiratória dos microrganismos pela interação com enzimas como a citocromo oxidase e succinase desidrogenase (Lok *et al*., 2006; Lok *et al*., 2007; Rai *et al*., 2009; Costa *et al*., 2010). De acordo com Allaker (2010), como o mecanismo de ação das nanopartículas de prata é multifatorial, diversas mutações precisam ocorrer para que os microrganismos consigam resistir aos seus efeitos. Entretanto, o uso destas nanopartículas deve ser feito com cautela, já que as mesmas podem induzir cito e genotoxicidade em células humanas, dependendo do tamanho e da concentração das partículas usadas (Carlson *et al*., 2008; AshaRani *et al*., 2009; Costa *et al*., 2010).

Panácek *et al*. (2009) verificaram que nanopartículas de prata exibiram efeito inibitório contra o crescimento de *C. albicans*, *C. parapsilosis* e *C. tropicalis* em concentrações tão baixas quanto 0,21 µg/mL. No estudo de Kim *et al*. (2008), a mínima concentração de nanopartículas de prata (1-7 µg/mL) que inibiu 80% do crescimento de *C. albicans* , *C. glabrata*, *C. parapsilosis*, *C. krusei*

e *Trichophyton mentagrophytes* foi similar à da anfotericina B (1-5 µg/mL) e inferior à do fluconazol (10-30 µg/mL).

Embora o efeito das nanopartículas de prata contra células planctônicas de espécies de *Candida* tenha sido relatado, sua efetividade antifúngica sobre os biofilmes destas espécies permanece incerta. Assim, o objetivo deste estudo foi avaliar a atividade antifúngica de nanopartículas de prata contra os biofilmes de *C. albicans* e *C. glabrata*, as quais estão etiologicamente relacionadas com as candidoses orais.

CAPÍTULO 1

Silver colloidal nanoparticles: antifungal effect against adhered cells and biofilms of *Candida albicans* and *Candida glabrata*[*]

[*] *Artigo publicado no periódico Biofouling*

Silver colloidal nanoparticles: antifungal effect against adhered cells and biofilms of *Candida albicans* and *Candida glabrata*

2.1. Abstract

The aim of this study was to evaluate the effect of silver nanoparticles (SN) against *Candida albicans* and *Candida glabrata* adhered cells and biofilms. SN (average diameter 5 nm) were synthesized by silver nitrate reduction with sodium citrate and stabilized with ammonia. Minimal inhibitory concentration (MIC) tests were performed for *C. albicans* (n = 2) and *C. glabrata* (n = 2) grown in suspension following the Clinical Laboratory Standards Institute microbroth dilution method. SN were applied to adhered cells (2 h) or biofilms (48 h) and after 24 h of contact their effect was assessed by enumeration of colony forming units (CFUs) and quantification of total biomass (by crystal violet staining). The MIC results showed that SN were fungicidal against all strains tested at very low concentrations (0.4-3.3 µg ml^{-1}). Furthermore, SN were more effective in reducing biofilm biomass when applied to adhered cells (2 h) than to pre-formed biofilms (48 h), with the exception of *C. glabrata* ATCC, which in both cases showed a reduction ~90%. Regarding cell viability, SN were highly effective on adhered *C. glabrata* and respective biofilms. On *C. albicans* the effect was not so evident but there was also a reduction in the number of viable biofilm cells. In summary, SN may have the potential to be an effective alternative to conventional antifungal agents for future therapies in *Candida*-associated denture stomatitis.

Keywords: silver; nanoparticles; biofilms; *Candida albicans*; *Candida glabrata*

2.2. Introduction

Candida-associated denture stomatitis is a common recurring inflammatory process that mainly involves the palatal mucosa of complete denture wearers (Jeganathan and Lin 1992). *Candida albicans* remains the most frequently isolated yeast in the oral cavity but other non-*C. albicans Candida* (NCAC) species have also been isolated and involved in disease (Webb et al. 1998; Ramage et al. 2004; Vanden Abbeele et al. 2008). Several authors (Coco et al. 2008; Vanden Abbeele et al. 2008) reported that the most frequent NCAC species isolated from dentures of elderly patients were *Candida glabrata* (44.1%) and *Candida tropicalis* (19.1%).

One attribute of virulence among *Candida* species is their ability to form biofilms that can develop on oral surfaces including mucosa and acrylic dentures (Hasan et al. 2009). These biofilms are frequently tolerant/resistant to the commonly used antifungal drugs including nystatin (Watamoto et al. 2009), amphotericin B (Hasan et al. 2009; Watamoto et al. 2009), fluconazole (Bagg et al. 2003; Hasan et al. 2009), itraconazole (Bagg et al. 2003), caspofugin (Watamoto et al. 2009), ketoconazole (Watamoto et al. 2009) and flucytosine (Watamoto et al. 2009). Therefore, these *Candida* biofilms, which are resistant to antifungal agents and host immune defenses, are often associated not only with chronic infections but also to failure of prostheses (Samaranayake et al. 2009). Consequently, novel strategies are needed to combat the emergence of antifungal resistance in general.

For many years, silver has been known for its significant broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi,

protozoa and certain viruses (Balazs et al. 2004), including antibiotic-resistant strains (Melaiye and Youngs 2005; Stobie et al. 2008). Silver, as antimicrobial agent, is used in wound dressings, burn treatments, creams and as coatings on different medical devices (Bjarnsholt et al. 2007; Monteiro et al. 2009). The antimicrobial activities of silver nanoparticles (SN) are related to their size and shape. Baker et al. (2005) observed that smaller particles with a larger surface area available for interaction have a higher bactericidal effect than larger particles. Moreover, Pal et al. (2007) found that triangular SN displayed greater biocidal action than rod or spherical nanoparticles. However, the use of SN must be undertaken with caution, because of its concentration-dependent toxicity (Carlson et al. 2008; AshaRani et al. 2009; Panácek et al. 2009). Carlson et al. (2008) found that SN with an average size of 15 nm and 30 nm exhibited significant cytotoxicity at 10-75 µg ml^{-1}, whereas 55 nm nanoparticles required a concentration of 75 µg ml^{-1} to promote a significant decrease in mammalian cell viability.

Antifungal activity of SN has been reported by some authors (Kim et al. 2008, 2009; Monteiro et al. 2009; Panácek et al. 2009; Rai et al. 2009). Panácek et al. (2009) found that SN prepared by the modified Tollens process exhibited inhibitory effect against *Candida* spp. at a concentration as low as 0.21 µg ml^{-1}. In the study of Kim et al. (2008), SN showed potent activity against clinical isolates and ATCC strains of *Trichophyton mentagrophytes* and *Candida* species (80% inhibitory concentration (IC_{80}), 1-7 µg ml^{-1}). The activity of SN was comparable to that of amphotericin B, but superior to that of fluconazole (amphotericin B IC_{80}, 1-5 µg ml^{-1}; fluconazole IC_{80}, 10-30 µg ml^{-1}) (Kim et al. 2008).

According to some researchers (Sondi and Salopek-Sondi 2004; Lok et al. 2006; Zheng et al. 2008; Kim et al. 2009), SN attach to the sulphur containing proteins of the cell membrane, thereby causing membrane damage and depleting the levels of intracellular ATP of the microorganism. Silver can also interact with the DNA of microorganisms, preventing cell reproduction (Damm et al. 2008). Moreover, Elechiguerra et al. (2005) observed that SN in the 1-10 nm range interacted with gp120 glycoprotein knobs, blocking the HIV-1 virus from binding to host cells.

Although the literature reports some studies related to the antifungal activity of SN, to the authors' knowledge, there are no studies concerning the effect of these particles against adhered cells and biofilms of *Candida* spp. Thus, the aim of the present study was to evaluate the effect of SN against adhered cells and biofilms of *C. albicans* and *C. glabrata* through quantification of the total biomass and cultivable cells. The hypothesis tested was that SN have an antifungal effect against sessile cells of *C. albicans* and *C. glabrata* and this capacity depends on the concentration of SN.

2.3. Materials and methods

Synthesis and characterization of silver colloidal nanoparticles

SN were synthesized by means of the Turkevich et al. (1951) method through the reduction of silver nitrate ($AgNO_3$) with sodium citrate ($Na_3C_6H_5O_7$), as detailed elsewhere (Monteiro et al. 2009), and both chemicals were obtained from Merck KGaA, Darmstadt, Hesse, Germany. The initial concentrations of the reaction components were 5 x 10^{-3} mol l^{-1} of $AgNO_3$ and 0.3 mol l^{-1} of $Na_3C_6H_5O_7$. The aqueous solutions of $AgNO_3$ and $Na_3C_6H_5O_7$ were kept at boiling temperature for ~6 min until the solution turned amber yellow. After that, 7.5 ml of a 1.4 mol l^{-1} solution of ammonia (NH_3) (Merck KGaA, Darmstadt, Hesse, Germany) were added. The formation of colloidal SN was confirmed by UV/Visible spectroscopy (Spectrophotometer Shimadzu MultSpec-1501, Shimadzu Corporation, Tokyo, Japan) and, later, by X-ray diffraction (XRD) (Diffractometer Rigaku DMax-2000PC, Rigaku Corporation, Tokyo, Japan). Transmission electron microscopy (TEM, Electron Microscope FEG-VP Supra 35, Carl Zeiss, Jena, Thüringen, Germany) was used in order to further characterize the synthesized SN.

Artificial saliva medium

Artificial saliva was prepared according to Lamfon et al. (2003). The composition per 1 l of deionized water was: 2 g of yeast extract (Liofilchem, Italy), 5 g of peptone (Liofilchem, Italy), 2 g of glucose (AppliChem, Germany), 1 g of mucin (Sigma–Aldrich, USA), 0.35 g of NaCl (AppliChem, Germany), 0.2 g of $CaCl_2$ (Riedel-de Haën, Germany) and 0.2 g of KCl (Pronalab, Portugal). The pH was adjusted with NaOH (Pronalab, Portugal) to 6.8.

Organisms and growth conditions

Two *Candida* species and two different strains of each species were used in this work. Regarding *C. albicans*, one strain was from the American Type Culture Collection, ATCC 10231, and the other was an oral clinical isolate (strain 324LA/94) from the culture collection of the Cardiff Dental School (Cardiff, UK). For *C. glabrata* one strain was from the American Type Culture Collection, ATCC 90030 and the other was an oral isolate (strain D1) obtained from the Biofilm Group of the Centre of Biological Engineering, University of Minho (Braga, Portugal). All *Candida* strains were subcultured on Sabouraud dextrose agar medium (SDA; Liofilchem, Italy) at 37ºC for 24 h. An inoculum of each yeast strain, obtained from SDA plates, was suspended in 30 ml of Sabouraud dextrose broth (SDB; Liofilchem, Italy) and incubated at 37ºC for 20-24 h under agitation (120 rpm). After incubation, the cells were harvested by centrifugation at 8000 rpm for 5 min at 15ºC. Thereafter, the pellet was washed twice with 30 ml of phosphate buffered saline (PBS; pH 7), and the yeasts were enumerated using a Neubauer counting chamber and adjusted to a concentration of 10^7 cells ml^{-1} in artificial saliva.

Minimum inhibitory concentration (MIC)

MIC was determined using the microdilution method in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI) (M27-A2). The colloidal suspension of SN was first diluted in deionized water in a geometric progression, from 2 to 1024 times. Then, each silver concentration obtained previously was diluted (1:5) in RPMI 1640 medium (Sigma-Aldrich, USA). The

final concentrations of SN in the dispersion ranged from 54 µg ml^{-1} to 0.1 µg ml^{-1} (54; 27; 13.5; 6.75; 3.38; 1.69; 0.84; 0.42; 0.21; 0.1 µg ml^{-1}). Inocula from 24 h yeast cultures on SDA were adjusted to a turbidity equivalent to a 0.5 McFarland standard in saline solution (0.85% NaCl). The yeast suspension was diluted (1:5) in saline solution and afterwards diluted (1:20) in RPMI 1640. Each yeast inoculum (100 µl) was added to the respective well of microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) containing 100 µl of each specific concentration of SN colloidal suspension. Controls devoid of SN were also included. The microtiter plates were incubated at 37ºC, and the MICs were determined visually as the lowest concentration of SN showing no yeast growth after 48 h. As a control, the influence of the stabilizers ($Na_3C_6H_5O_7$ and NH_3) used for synthesizing the SN was also tested. For that, the solution was diluted in RPMI 1640 medium in a microtiter plate to the same concentrations used when diluting the suspension of silver colloidal nanoparticles. All assays were repeated in duplicate on three different occasions.

Application of SN in adhered cells and biofilms

Adhesion assay

An aliquot of yeast cell suspension (200 µl of 10^7 cells ml^{-1} in artificial saliva) was added to each well of a 96-well microtiter plate and incubated for 2 h at 37ºC in an orbital shaker incubator at 120 rpm. After this, the cell suspensions were aspirated, and each well washed once with 200 µl of PBS to remove loosely adherent cells.

Biofilm formation

Candida biofilms were developed in the 96-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) using the methodology described by Silva et al. (2010). Cell suspensions (200 µl of 1 x 10^7 cells ml^{-1} in artificial saliva) were pipetted into each well and incubated for 48 h at 37ºC in a shaker at 120 rpm. After 24 h, 100 µl of artificial saliva medium were removed and an equal volume of fresh artificial saliva was added. After biofilm formation for 48 h, the medium was aspirated and each well was rinsed once with 200 µl of PBS to remove non-adherent cells.

Treatment with SN

In this study, SN were added to adhered yeast cells (2 h) and to mature biofilms (48 h) and their effect was assessed after contact for 24 h. Stock suspensions of silver colloidal nanoparticles were serially diluted with deionized water and RPMI 1640 to obtain concentrations ranging from 54 to 0.1 µg ml^{-1}, similar to the procedure for MIC determination. Each dilution (200 µl) was added to the columns of a 96-well microtiter plate, in decreasing concentrations, containing adhered cells or biofilms (prepared as described previously). For controls, a column of each plate was handled in an identical way except that no SN suspension was added. The plates were incubated at 37ºC for 24 h in an orbital shaker incubator at 120 rpm. After treatment with SN, adhered cells and biofilms were washed once with PBS to remove loosely attached cells prior to analysis of biomass and cultivable cells.

All assays were performed in triplicate and on three separate occasions.

Adhered cells and biofilm quantification

Biomass quantification by crystal violet staining

For fixation of the adhered cells and biofilms, 200 µl of 99% methanol (Romil, UK) were added to each well (containing adhered cells or biofilms treated with SN, as described previously), after 15 min the methanol was removed and the plates were allowed to dry at room temperature. Then, 200 µl of crystal violet stain (CV; 1%, v/v) (Merck, Germany) were added to all wells. After 5 min, the excess of CV was removed and the plates were gently washed in water. Finally, 200 µl of acetic acid (33%, v/v) (Pronalab, Portugal) were added to all wells to dissolve the CV stain and the absorbance was measured at 570 nm. The assays were performed in triplicate and on three separate occasions.

Quantification of cultivable cells

The walls of each well, containing adhered cells or biofilms treated with SN, were scraped and the suspensions obtained were vigorously vortexed for 5 min to disaggregate cells. Serial decimal dilutions (in PBS) were plated on SDA. Agar plates were incubated for 24 h at 37ºC, and the total number of colony forming units (CFUs) per unit area (Log_{10} CFU cm^{-2}) of microtiter plate well were enumerated. The assays were performed on three separate occasions.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS-Statistical Package for the Social Sciences, Inc., Chicago, USA) and the results were compared using

a one-way ANOVA followed Bonferroni test. The significance level was set at $P < 0.05$.

2.4. Results

Synthesis and characterization of silver colloidal nanoparticles

In this work, the silver colloidal nanoparticles were stabilized using NH_3 in order to prevent aggregation. NH_3 plays an important growth moderating role, making it possible to stabilize metallic silver nanoparticles, since free silver ions, which are responsible for particle growth and the formation of new nuclei, are trapped by the formation of diammine silver (I) complexes (Gorup et al. 2011). The absorption spectrum of the colloidal suspension displayed in Figure 1A shows a well-defined plasmon band centered at 430 nm, characteristic of nanosized silver. Indeed, the symmetrical shape of the plasmon band in Figure 1A confirms the colloidal stability and sharp particle size distribution. The TEM image (Figure 1B) of colloidal SN revealed the presence of nearly spherical particles with average size of 5 nm. Moreover, the concentration of the resultant colloidal suspension was determined to have 540 µg of Ag ml^{-1}. The characteristic XRD pattern (Figure 1C) also confirmed the presence of metallic SN, with a cubic crystalline structure (JCPDS 04-0783). The diffraction peaks assigned with Ag at 38.5º, 44.5º, 64.8º and 78º can be attributed to the (1 1 1), (2 0 0), (2 2 0) and (3 1 1) crystallographic planes of metallic Ag, respectively.

Minimum inhibitory concentration (MIC)

The results showed that the SN were fungicidal against all of the tested yeasts at very low concentrations and the fungicidal activity was dependent on the yeast species and strains tested (Table 1). These results were confirmed by plating the content of each well on SDA, and there was no growth for any of the strains

resultant from the MIC point. The lowest MICs of SN, at a silver concentration of 0.4-0.8 µg ml^{-1}, were obtained against *C. albicans* 324LA/94 and *C. glabrata* ATCC 90030. On the other hand, *C. albicans* ATCC 10231 and *C. glabrata* D1 were less sensitive, with MIC values equal to 0.8-1.6 µg ml^{-1} and 1.6-3.3 µg ml^{-1}, respectively. The control solution without silver ($Na_3C_6H_5O_7$ + NH_3) did not reveal any effect on the tested yeasts.

Effect of SN on biofilm biomass

Figure 2A presents the results of biomass reduction of biofilms formed 24 h after the application of SN to adhered cells. These data show that SN were effective in inhibiting biofilm development by all the tested yeasts. When compared to the control group (biofilms without SN), *C. albicans* ATCC 10231 showed significant biomass reduction (~60%) at silver concentrations > 1.6 µg ml^{-1} ($P < 0.001$), while for *C. albicans* 324LA/94 ($P = 0.009$), *C. glabrata* ATCC 90030 ($P < 0.001$) and *C. glabrata* D1 ($P < 0.001$) this effect was only significant for silver concentrations > 3.3 µg ml^{-1}. In general, *C. glabrata* strains showed a higher percentage of biomass reduction (> 90%) for silver concentrations above 3.3 µg ml^{-1} than *C. albicans* strains (Figure 2A) and independently of the silver concentration increase. However, *C. albicans* ATCC 10231 and *C. albicans* 324LA/94 showed a higher silver concentration dependent biomass reduction, rising to ~85% above a silver concentration of 6.7 µg ml^{-1} (Figure 2A).

Interestingly, SN had a similar effect in reducing the biofilm biomass of *C. glabrata* strains when applied to adhered cells (2 h), due to inhibition of biofilm formation, and on pre-formed biofilms (48 h), which in this case represents a true

biomass reduction. However, on *C. albicans*, silver particles were more effective when applied to adhered cells (Figure 2A and B). Furthermore, biomass reduction of *C. glabrata* ATCC 90030 (Figure 2B) was statistically significant only for the highest SN concentration (reduction of 97.12%; $P = 0.028$).

Effect of SN on biofilm cultivable cells

Mean and standard deviation (SD) values of the $\log_{10}$ CFU cm^{-2} obtained in the two experimental conditions tested for each *Candida* strain are shown in Figure 3.

After contact of the adhered cells with SN for 24 h a reduction in the number of cells was observed, expressed as $\log_{10}$ CFUs, dependent on the silver concentration (Figure 3A), with the exception of *C. albicans* ATCC 10231. The other yeast strains, *C. albicans* 324LA/94, *C. glabrata* ATCC 90030, and *C. glabrata* D1 showed a significant reduction in the number of CFUs for silver concentrations in the range 3.3 µg ml^{-1} to 54 µg ml^{-1} (Figure 3A). However, *C. glabrata* biofilm cells were more sensitive to silver than those of *C. albicans* 324LA/94. At a silver concentration of 3.3 µg ml^{-1} there was a 1.5-$\log_{10}$, 6.5- $\log_{10}$ and 4.0- $\log_{10}$ reduction in the number of CFUs for *C. albicans* 324LA/94 ($P = 0.003$), *C. glabrata* ATCC 90030 ($P < 0.001$) and *C. glabrata* D1 ($P < 0.001$), respectively, compared to the control values. These findings agree with the results of the CV staining assay which also show a significant biomass reduction at a silver concentration of 3.3 µg ml^{-1} (Figure 2A). For *C. albicans* ATCC 10231, although there was an approximately 1.1-$\log_{10}$ reduction in the number of CFUs at a silver concentration of 6.7 µg ml^{-1}, this difference was not statistically significant when compared to the control group ($P = 0.496$; $P > 0.05$).

Figure 3B displays the number of 24 h biofilm cultivable cells after contact with different SN concentrations for pre-formed biofilms (48 h). Although at high silver concentration (54 µg ml^{-1}), there was a reduction in the log_{10} of the mean number of CFUs of 1.73-log_{10} for *C. albicans* ATCC 10231 (*P* = 0.112), 1.73-log_{10} for *C. albicans* 324LA/94 (*P* = 0.800) and 1.87-log_{10} for *C. glabrata* D1 (*P* = 0.055), these reductions were not significant when compared to the control groups. However, at the same silver concentration, *C. glabrata* ATCC 90030 biofilms showed a significant log_{10} CFU reduction compared to the control group (3.39-log_{10} reduction; *P* = 0.003). Finally, the comparison of biofilm biomass (Figure 2B) and biofilm cultivable cells (Figure 3B) clearly shows that the highest reductions in total biomass and number of log_{10} CFUs occurred for *C. glabrata* ATCC 90030 at the silver concentration of 54 µg ml^{-1}.

2.5. Discussion

The present study evaluated the effect of SN against adhered cells and biofilms of *C. albicans* and *C. glabrata*, through quantification of their biomass and cultivable cells. The research hypothesis was accepted since SN showed activity against the tested yeasts and this capacity was dependent on the silver concentration.

In the MIC tests, performed according to CLSI methodology (Table 1), both strains from each species were susceptible to SN. SN promoted 100% reduction in the planktonic growth of the yeasts in concentrations ranging from 0.4 µg ml^{-1} to 3.3 µg ml^{-1}. Panácek et al. (2009) observed that the lower MIC values for *C. albicans* occurred at a SN concentration of 0.052 µg ml^{-1} to 0.1 µg ml^{-1}, while Kim et al. (2008) reported values of 2-4 µg ml^{-1} for *C. albicans* and 1-7 µg ml^{-1} for *C. glabrata*. These differences may be explained by differences in the nanoparticle synthesis methods. Kim et al. (2008) dissolved solid silver in nitric acid and the Ag^{+} ions were reduced by sodium chloride, while Panácek et al. (2009) used a modified Tollens reaction to prepare SN, in which Ag^{+} ions are reduced by saccharides in the presence of ammonia. Subsequent stabilization of SN by sodium dodecyl sulfate was performed in the study of Panácek et al. (2009). In addition, the differences in the MICs of the SN probably result from differences in the strains tested.

Nevertheless, SN had already been shown to be effective against planktonic *Candida* cells, so the more important question was whether treatment with these particles could eradicate adhered cells and inhibit mature biofilms.

Regarding the results obtained with the CV staining and CFU enumeration assays for adhered cells (Figures 2A and 3A), in general, a significant reduction in biomass and in the number of CFUs was observed for silver concentrations at or higher than 3.3 µg ml^{-1}. Therefore, for all tested strains, SN showed an ability to inhibit biofilm formation when applied to already adhered cells. SN present a large surface area, which provides better contact with microorganisms (Rai et al. 2009). According to Kim et al. (2009), SN affect yeast cells by attacking their membranes, thus disrupting the membrane potential. These authors observed, by transmission electron microscopy, the formation of 'pits' on the membrane surfaces of *C. albicans* and finally the formation of pores and subsequent cell death. Moreover, the effective silver concentration found in the present study was lower than in previous reports concerning the toxic concentration of SN *in vitro* against human cells (Carlson et al. 2008; Panácek et al. 2009).

Another interesting observation from the present study is that SN were more effective in reducing total biomass and CFUs when applied to adhered cells (2 h) (Figures 2A and 3A), than to pre-formed biofilms (48 h) (Figure 3A and 3B). Such results are in good agreement with those published by Chandra et al. (2001), who found that the progression of drug resistance in *C. albicans* biofilms was associated with the concomitant increase in metabolic activity of developing biofilms. When compared to young and mature *Candida* biofilms of 24-48 h, the adhesion phase contains a lower cell mass (Seneviratne et al. 2009), due to the incipient production of the extracellular matrix, one of the most specific traits of biofilms. Nevertheless, adhesion phase cells seem to be in a metabolically excited state compared with their older counterparts (Seneviratne et al. 2009) in well

established biofilms. This may indicate that the observed decrease in susceptibility to SN was a reflection of a lower metabolic activity of cells in mature biofilms.

However, the experiments on pre-formed biofilms (Figure 3B) showed that at the highest silver concentration (54 µg ml^{-1}) there was a reduction in the log_{10} of the mean number of CFUs for all *Candida* strains, but this reduction was only significant for *C. glabrata* ATCC 90030, when compared to the control group (without silver). This effect of SN on pre-formed biofilms may be due to the presence of water channels throughout the biofilm. Since water channels are present in all biofilms for nutrient transportation, SN may directly diffuse inside the matrix layer through the pores and may impart an antifungal function (Kalishwaralal et al. 2010).

Despite the reduction in the number of CFUs at the highest silver concentration (Figure 3B), it was found that a progressive increase in silver concentration did not provide significant reductions in the number of CFUs, characterizing a relatively weak dose-response. Perhaps increasing the silver concentration above 54 µg ml^{-1} would result in a better dose-response. Furthermore, when compared with the antifungal drug concentrations used in some studies, the silver concentrations tested in this study can be considered low. Vandenbosch et al. (2010) evaluated the fungicidal activity of miconazole against mature *Candida* biofilms and found that a 24 h-treatment with miconazole resulted in a significant reduction (ranging from 89.3% to 99.1%) in the number of CFUs for all strains investigated (*C. albicans*, *C. glabrata*, *C. Krusei*, *C. parapsilosis* and *C. tropicalis*). However, it should be noted that the antifungal

concentration used in this study (2081 µg ml^{-1}) was higher than the common concentration used *in vivo*. Tobudic et al. (2010) verified that posaconazole at concentrations of 2 and 256 µg ml^{-1} failed to significantly reduce the CFUs of biofilms of *C. albicans*, compared with the untreated control (< 1 log_{10} CFU ml^{-1}). However, the combination of 1 µg ml^{-1} of amphotericin B and 2 µg ml^{-1} of posaconazole showed the greatest decrease in the CFUs (> 2 log_{10} CFU ml^{-1}). Thus, the most traditional antifungals applied alone seem to have a low effect against biofilms of *Candida* species compared to the effect of SN as observed in the present study.

With regard to biomass reduction in pre-formed biofilms (Figure 2B), the SN were effective only for *C. glabrata* strains. *C. albicans* forms larger and more complex biofilms than *C. glabrata* (Samaranayake et al. 2005). In the studies of Samaranayake et al. (2005) and Seneviratne et al. (2010), *C. glabrata* biofilms presented reduced thickness, were less profuse, and were devoid of hyphal elements, when compared with *C. albicans* biofilms. The morphogenic transition of yeast to hyphae has been shown to play an important role in biofilm formation in *C. albicans* (Samaranayake et al. 2005). Probably, hyphal elements facilitate the exuberant architecture of *C. albicans* (Seneviratne et al. 2009) biofilms, making them more difficult to eliminate. Moreover, a relationship between biofilm thickness and resistance to antibiotics has been observed (Mah and O'Toole 2001). Thus, all these factors may explain the higher activity of SN against *C. glabrata* biofilms, which is still of major importance since this species is known to be very resistant to common antifungal agents, making it very difficult to eliminate.

Several mechanisms have been proposed to explain the resistance of *Candida* biofilms to antifungal agents, viz. the robust biofilm architecture, a decreased metabolic activity, altered gene expression, the extracellular matrix, the presence of 'persister cells' and higher anti-oxidative capacities (Mah and O'Toole 2001; Ramage et al. 2005; Seneviratne et al. 2010). Harrison et al. (2006) suggest that *Candida* biofilms may adsorb metal cations from their surroundings and that sequestration in the extracellular matrix may contribute to resistance. However, the exact mechanism by which fungi in the biofilm mode can acquire resistance remains to be elucidated.

Finally, in clinical terms, the *in vitro* data obtained in the present study demonstrate that SN may have an important role in preventing *Candida* biofilm formation in the oral cavity. However, more studies are required to investigate the morphology and the matrix composition of *Candida* biofilms in the presence of SN. Further studies on the development, the antifungal properties, the cytotoxicity and genotoxicity of SN with different sizes and with stabilizing agents are also necessary for *in vivo* experimentation with such antifungal agents. Such studies would stimulate the development of antifungal drugs based on SN working to prevent *Candida*-associated denture stomatitis.

2.6. Conclusions

SN exhibit fungicidal activity against all the tested yeasts at very low concentrations (0.4-3.3 μg ml^{-1}). These nanoparticles were more effective in inhibiting biofilm formation than in controlling mature biofilms. However, their antifungal activity was higher against *C. glabrata* than against *C. albicans*. The significance of this work is that this is the first report concerning the effect of SN against adhered cells and biofilms of *Candida* spp. Moreover, the fact that the SN used here can easily be prepared in a cost-effective manner is also an important factor. Thus, it can be expected that SN may have potential as an antifungal agent to prevent *Candida*-associated denture stomatitis.

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Table 1. Minimum inhibitory concentrations (MIC) of silver nanoparticles against the tested yeasts

Tested yeasts	**MIC (µg ml^{-1})**
C. albicans ATCC 10231	0.8-1.6
C. albicans 324LA/94	0.4-0.8
C. glabrata ATCC 90030	0.4-0.8
C. glabrata D1	1.6-3.3

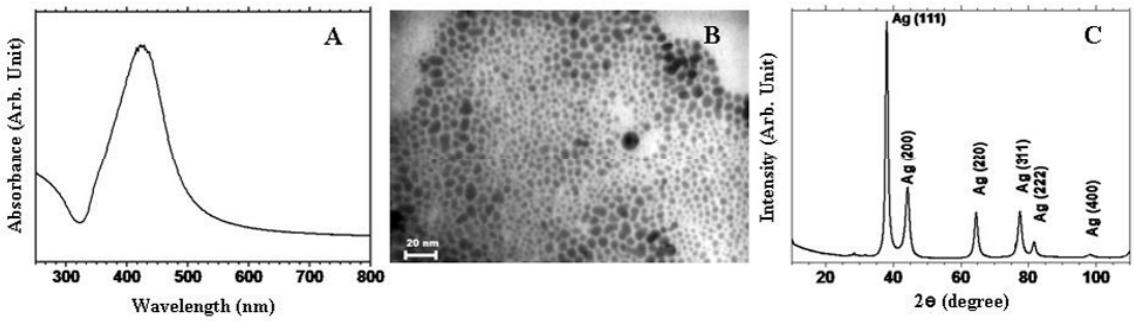

Figure 1. (A) UV-Vis absorption spectrum; (B) transmission electron microscope image (magnification: 880 KM); (C) X-ray diffraction pattern of SN synthesized through the reduction of silver nitrate with sodium citrate.

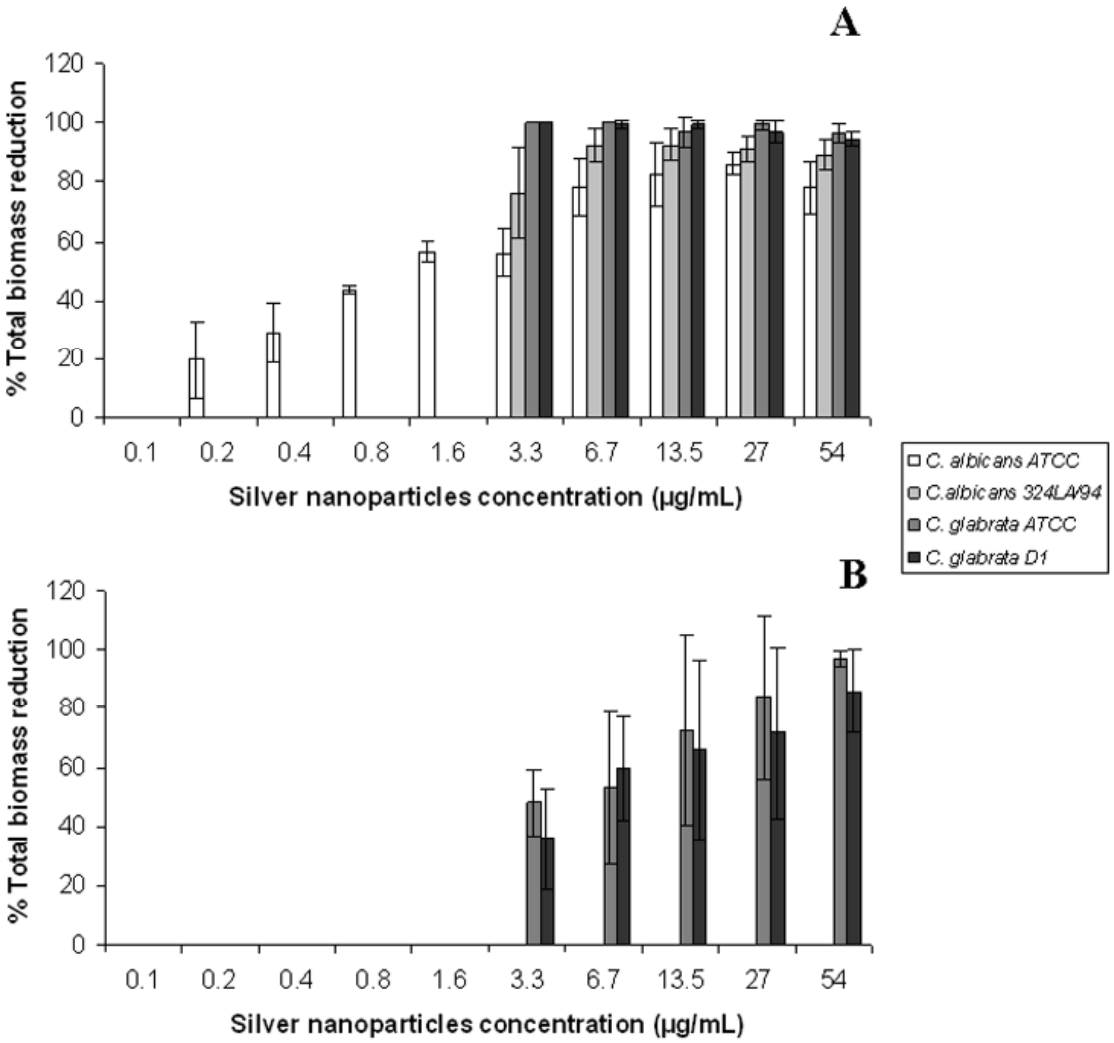

Figure 2. (A) Percentage of total biomass reduction of adhered cells of *C. albicans* and *C. glabrata* obtained with the CV staining assay; (B) Their mature biofilms after treatment for 24 h with different SN concentrations. Error bars indicate the SDs of the means. Note: there was no total biomass reduction in pre-formed biofims of *C. albicans* strains.

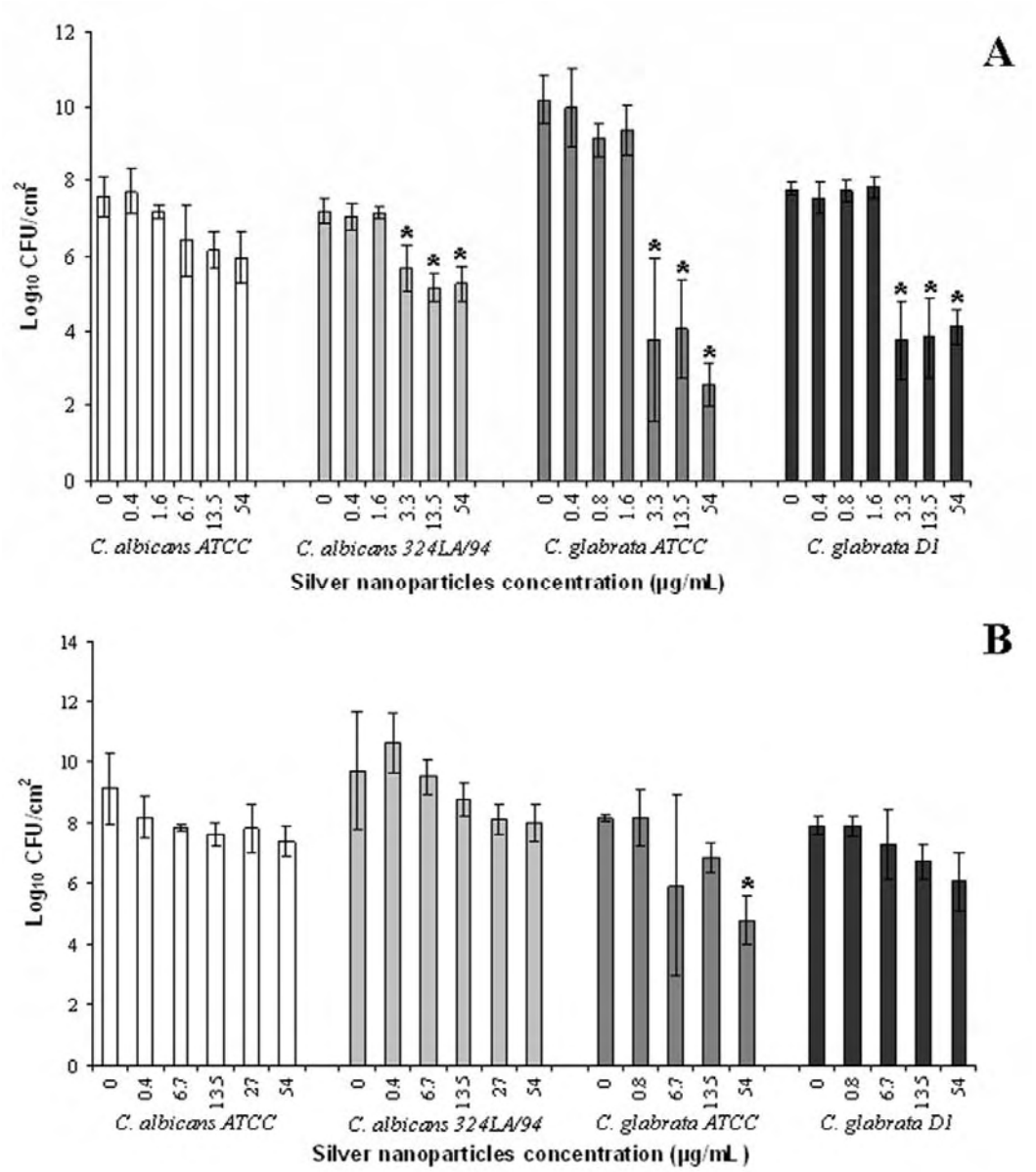

Figure 3. Mean values of the logarithm of colony forming units normalized by area of adhesion (log_{10} CFU cm^{-2}) of adhered cells of *C. albicans* and *C. glabrata* (A), and biofilms (B) after treatment for 24 h with different SN concentrations. Error bars indicate the SDs of the means. *Indicates $P < 0.05$, as compared to the control group, using a one-way ANOVA with the Bonferroni test.

CAPÍTULO 2

Silver nanoparticles: influence of stabilizing agent and diameter on antifungal activity against *Candida albicans* and *Candida glabrata* biofilms[*]

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Silver nanoparticles: influence of stabilizing agent and diameter on antifungal activity against *Candida albicans* and *Candida glabrata* biofilms

3.1. Abstract

Aim: The purpose of this work was to evaluate the size-dependent antifungal activity of different silver nanoparticles (SN) colloidal suspensions against *Candida albicans* and *Candida glabrata* mature biofilms. **Methods and Results:** The research presented herein used SN of three different average sizes (five, 10 and 60 nm), which were synthesized by the reduction of silver nitrate through sodium citrate and which were stabilized with ammonia or polyvinylpyrrolidone. Minimal inhibitory concentration (MIC) assays were performed using the microdilution methodology. The antibiofilm activity of SN was determined by total biomass quantification (by crystal violet staining) and colony forming units enumeration. MIC results showed that all SN colloidal suspensions were fungicidal against the tested strains at very low concentrations (0.4-3.3 μg ml^{-1}). With regard to biomass quantification, SN colloidal suspensions were very effective only against *C. glabrata* biofilms, achieving biomass reductions around 90% at a silver concentration of 108 μg ml^{-1}. In general, all SN suspensions promoted significant log_{10} reduction of the mean number of cultivable biofilm cells after exposure to silver concentrations at or higher than 108 μg ml^{-1}. Moreover, the results showed that the particle size and the type of stabilizing agent used did not interfere in the antifungal activity of SN against *Candida* biofilms. **Conclusion:** This study suggests that SN have antifungal therapeutic potential, but further studies are still required namely regarding formulation and delivery means. **Significance and Impact of Study:** SN may contribute to the development of new strategies for the improvement of oral health and quality of life particularly of the complete denture wearers.

Keywords: biofilms, *Candida albicans*, *Candida glabrata*, infection, silver nanoparticles.

3.2. Introduction

The emergence of a pathogenic state known as denture stomatitis, which is associated with biofilm formation by *Candida* species, particularly *Candida albicans* and *Candida glabrata* (Coco *et al*. 2008) is common in complete denture wearers. *Candida* infections display increased resistance to antifungal therapy, leading to treatment failure and to recalcitrant infections (Watamoto *et al*. 2009). This fact has stimulated the possibility of using silver nanoparticles (SN) to control *Candida* biofilm formation.

The chemical reaction method is one of the most employed and inexpensive methods to synthesize SN. It is based on the use of a reducing agent (e.g, sodium citrate) to reduce Ag^{+} to Ag^{0}, and a stabilizer (e.g., ammonia (NH3), polyvinylpyrrolidone (PVP)) to control particle growth and prevent aggregation (Kvítek *et al*. 2008; Gorup *et al*. 2011; Monteiro *et al*. 2012). The SN action mechanism against bacterial and fungal cells is relatively well described (Monteiro *et al*. 2009), and as, according to Allaker (2010), silver may act on a broad range of microbial targets, many mutations need to occur for microorganisms to resist its antimicrobial effect. Moreover, an inverse relationship between SN size and antimicrobial activity has been demonstrated by Baker *et al*. (2005), who reported that smaller particles, with a larger surface area available for interaction with planktonic microbial cells, were more effective than larger particles.

Although the activity of SN against *C. albicans* and *C. glabrata* adhered cells and biofilms was demonstrated recently (Monteiro *et al*. 2011), research regarding the susceptibility of *Candida* biofilms to SN of different sizes, and

synthesized with different stabilizing agents, is unknown at the moment. For this reason, the current study investigated, through the quantification of total biomass and cultivable cells, the size-dependent antifungal activity against *C. albicans* and *C. glabrata* biofilms of SN synthesized with two different stabilizing agents. The null hypotheses were that: (a) there would be no difference among the different sizes of SN in decreasing total biomass and cultivable cells of *Candida* biofilms, and (b) the type of stabilizing agent would not interfere with SN efficacy.

3.3. Materials and methods

Synthesis and characterization of silver colloidal nanoparticles

For the synthesis of silver colloidal nanoparticles, silver nitrate ($AgNO_3$), sodium citrate ($Na_3C_6H_5O_7$) and ammonia (NH_3) were provided by Merck KGaA (Darmstadt; Germany) and polyvinylpyrrolidone (PVP) was obtained from Sigma-Aldrich (St Louis, MO). SN of three different sizes: 5 (SN5), 10 (SN10) and 60 (SN60) nm, and stabilized with NH_3 (N) or PVP (P) were prepared according to the procedure reported by Gorup *et al*. (2011). Briefly, 7.5 ml of a solution of sodium citrate at 0.3 mol l^{-1} and 5 drops of ammonia (1.4 mol l^{-1}) were added to a tri-neck flask containing 150 ml of an aqueous solution of $AgNO_3$ at 5.0 x 10^{-3} mol l^{-1} under the temperature of 90ºC. The solution was kept stirring at 90ºC until it turned yellow, indicating the formation of SN. The silver colloidal nanoparticles were stabilized by adding 7.5 ml of a solution with 1.4 mol l^{-1} of NH_3. The new solution was kept stirring and heating for two more minutes, and the suspension was then allowed to cool at room temperature. In another flask, 1 ml of a solution of 102 g l^{-1} of PVP was added to the colloidal silver after lowering of the temperature. In order to characterize the silver colloidal nanoparticles suspensions, UV/Visible absorption spectroscopy (Spectrophotometer Shimadzu MultSpec-1501, Shimadzu Corporation, Tokyo, Japan) and Transmission Electron Microscopy (TEM, Electron Microscope FEG-VP Supra 35, Carl Zeiss, Jena, Thüringen, Germany) were performed.

Yeast strains and growth conditions

The nutrient source for biofilm formation was artificial saliva, used to mimic the in vivo oral cavity conditions. It was prepared according to Lamfon *et al*. (2003) and its composition per 1 l of deionized water was: 2 g of yeast extract (Liofilchem, Roseto degli Abruzzi, Italy), 5 g of peptone (Liofilchem), 2 g of glucose (AppliChem, Darmstadt, Germany), 1 g of mucin (Sigma-Aldrich), 0.35 g of NaCl (AppliChem), 0.2 g of $CaCl_2$ (Riedel-de-Haën, Seelze, Germany) and 0.2 g of KCl (Pronalab, Lisbon, Portugal). The pH was adjusted with NaOH (Pronalab) to 6.8.

Candida strains used for the present experiments were *C. albicans* 324LA/94 (provided by 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), both being oral clinical isolates. Yeasts were first subcultured on Sabouraud dextrose agar medium (SDA; Liofilchem, Italy) at 37º C for 24 h. A cellular suspension of each yeast strain was prepared in Sabouraud dextrose broth (SDB; Liofilchem, Italy) medium and incubated overnight at 37ºC and 120 rpm. Afterwards, the cells were harvested by centrifugation (6500 g for 5 min at 15ºC), washed twice in phosphate buffered saline (PBS; pH 7) and adjusted to a concentration of 10^7 cells ml^{-1} in artificial saliva, using a Neubauer counting chamber.

Determination of minimum inhibitory concentrations (MICs)

MICs were determined for the six colloidal suspensions of SN against both *Candida* strains in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI) (M27-A2), as detailed in a previous study (Monteiro *et al*. 2011).

Biofilm formation and treatment with SN

Mature *Candida* biofilms aged 48 h were formed in 96-well microtiter plates according to Silva *et al*. (2010). Briefly, 200 µL of *Candida* cell suspension (1 x 10^7 cells ml^{-1} in artificial saliva) were placed into each well and incubated at 37ºC under 120 rpm. Artificial saliva medium was renewed after 24 h. Following *Candida* biofilm formation (48 h), the medium was aspirated and non-adherent cells were removed by washing with 200 µl of PBS. Next, RPMI 1640 medium (200 µl) containing different concentrations (0.4, 1.6, 6.7, 13.5, 27, 54, 108 and 216 µg ml^{-1}) of each SN suspension was then added to the pre-formed biofilms. The microtiter plates were incubated at 37º C for 24 h in an orbital shaker incubator at 120 rpm. After treatment with SN, biofilms were washed once with PBS to remove loosely attached cells. Controls devoid of silver were included in the assay. All assays were performed independently three times in triplicate.

Biofilm quantification

Biomass of *Candida* biofilms treated with SN was evaluated by the crystal violet (CV) staining method. Initially, 200 µl of 99% methanol (Romil, Cambridge, UK)

were added to the biofilms for 15 min to allow cell fixation. After, methanol was removed and the microtiter plates were dried at room temperature. Then, 200 µl of CV stain (1% v/v) (Merck, Germany) were added into the wells containing biofilms previously fixed. After 5 min, the excess of CV was removed and the plates were washed with deionized water and allowed to dry at room temperature. Afterwards, 200 µl of acetic acid (33% v/v) (Pronalab, Portugal) were pippeted into each well in order to dissolve and remove the CV stain from the biofilms. Finally, absorbance was measured at 570 nm. The assays were performed in triplicate and on three separate occasions.

For cultivable counts of *Candida* biofilm cells exposed to SN, biofilms were scraped from the wells and the suspensions obtained were vigorously vortexed for 5 min to disaggregate cells. Serial dilutions (in PBS) of each biofilm cell suspension were prepared, plated on SDA, and the plates were incubated at 37ºC. After 24 h, the total number of colony forming units (CFUs) per unit area (Log_{10} CFU/cm^2) of microtiter plate well were enumerated. For each condition studied, three separate experiments were performed.

Statistical analysis

Biofilm biomass and cultivable cells of the treated groups with SN were analysed using analysis of variance (ANOVA) followed by Bonferroni test. Statistical analyses were performed with the SPSS software (SPSS -Statistical Package for the Social Sciences, Inc., Chicago) with significance of 5%.

3.4. Results

Silver colloidal nanoparticles characterization

Silver nanoparticles at 540 µg ml^{-1} with different diameters were formed through the reduction of $AgNO_3$ with $Na_3C_6H_5O_7$ followed by stabilization with NH_3 (N) or PVP (P). These procedures led to six different colloidal suspensions of SN, depending on the stabilizing agent used and the particle size: SN5-N, SN5-P, SN10-N, SN10-P, SN60-N, and SN60-P. In the UV/Visible analysis (Fig. 1), the absorption peak of SN ranged from 430 to 460 nm, characterizing the nanosized silver particles. The absorption spectra of the SN5-N, SN5-P, SN10-N, and SN10-P colloidal suspensions (Fig. 1aI, 1aII, 1bI and 1bII) demonstrated well-defined plasmon bands centered at 430 nm. In the case of SN60-N and SN60-P (Fig. 1cI and 1cII), the absorption peaks were attained at approximately 460 nm, possibly due to the larger size of the particles. The symmetrical shapes of all plasmon bands in Fig. 1 confirmed the colloidal stability and sharp particle size distribution. These bands have been commonly assigned to nanoparticles having a spherical or spheroidal shape. TEM observations (Fig. 2) indicated that the nanoparticles were well formed, nearly spherical and dispersed, and with mean diameters of 5 (Fig. 2aI and 2aII), 10 (Fig. 2bI and 2bII) and 60 nm (Fig. 2cI and 2cII). Furthermore, it can be noted a few agglomerates of particles mainly when stabilized by PVP (Fig. 2bII and 2cII).

Minimum inhibitory concentration

MICs of the six SN colloidal suspensions determined against planktonic cells of *Candida* are shown in Table 1. It was possible to observe that the two species of *Candida* were susceptible to all the SN tested in very low concentrations. The lowest MIC values were against *C. albicans* 324LA/94, using SN5-N, SN60-N and SN60-P (0.4-0.8 µg ml^{-1}). Additionally, between the two *Candida* spp. tested, *C. glabrata* D1 was the least susceptible to SN, with MIC values ranging from 0.8-3.3 µg ml^{-1}. With regard to the particle size and the stabilizing agents, interestingly, MIC values for each *Candida* species remained almost the same. Solutions of $Na_3C_6H_5O_7$, NH_3, and PVP, used as controls did not exhibit any antifungal activity on the *Candida* spp. assayed.

Biofilm quantification

The results displayed in Fig. 3a show that SN were not effective in reducing total biomass of *C. albicans* 324LA/94 biofilms. Interestingly, it was possible to note that, at concentrations ranging from 0.4 to 108 µg ml^{-1}, all SN colloidal suspensions promoted an increase in biofilm biomass regardless of the silver concentration tested, except for SN5-N. When compared to the control values, the biomass increase was significant for SN10-N at concentrations of 1.6 (P = .001), 6.7 (P = .003), 13.5 (P = .004), 27 (P = .019) and 216 µg ml^{-1} (P = .002), and for SN60-N at a concentration of 216 µg ml^{-1} (P = .032). Curiously, the comparisons of different colloidal suspensions at the same silver concentration showed no statistical differences (P > .05).

However, on *C. glabrata* D1 (Fig. 3b), all SN colloidal suspensions were very effective in reducing total biomass after 24h of treatment of mature biofilms (formed for 48h). When compared to the control groups (biofilms without SN) SN5-N, SN5-P, SN10-N, SN10-P, SN60-N and SN60-P showed significant biomass reduction at silver concentrations greater than or equal to 1.6 (P = .001), 6.7 (P = .011), 13.5 (P = .002), 6.7 (P = .001), 1.6 (P = .011) and 6.7 µg ml^{-1} (P < .001), respectively. In general, *C. glabrata* D1 demonstrated a biofilm biomass reduction dependent on silver concentration, achieving biomass reductions greater than 50% at a concentration of 13.5 µg ml^{-1} and around 90% at a concentration of 108 µg ml^{-1}, for all colloidal suspensions tested.

Regarding to the effect of SN on biofilm cultivable cells, Fig. 4 presents mean values and standard deviations of log_{10} CFU/cm^2 for both *Candida* species. For *C. albicans* 324LA/94 (Fig. 4a), after 24h of biofilms treatment with SN colloidal suspensions, it was noted that the smaller particles (SN5-N and SN5-P) had the antifungal activity significantly increased from a concentration of 27 (P = .006) and 13.5 µg ml^{-1} (P < .015), respectively. In addition, using SN5-N and SN5-P at a silver concentration of 216 µg ml^{-1} there was 3.51-log_{10} (P < .001) and 3.36-log_{10} (P < .001) reduction in the number of CFUs, respectively, compared to the control groups. However, SN10-N similarly to SN10-P, and SN60-N similarly to SN60-P, started significant decrease in the number of CFUs in concentrations over 54 (P = .002; P = .007) and 27 µg ml^{-1} (P = .027; P = .015), respectively, suggesting the type of stabilizing agent was not decisive for antifungal activity against *C. albicans* 324LA/94 biofilms. Comparing the different SN colloidal

suspensions within the same silver concentration, no significant statistical differences ($P > .05$) were found in the average number of $\log_{10}$ CFUs.

For *C. glabrata* D1 (Fig. 4b), when each SN colloidal suspension was evaluated separately and compared to the control groups, the data showed that the SN5-N ($P = .015$) and SN10-P ($P < .010$) colloidal suspensions induced a significant decrease in the number of biofilm cells at silver concentrations above 54 µg ml^{-1} while for SN5-P ($P < .001$), SN10-N ($P = .006$), SN60-N ($P = .010$) and SN60-P ($P = .009$) this activity was statistically significant for silver concentrations greater than or equal to 27 µg ml^{-1}. SN5-P and SN60-P at silver concentration of 216 µg ml^{-1} exhibited the highest reductions in the average number of CFUs, with reductions of 4.24-$\log_{10}$ ($P < .001$) and 3.72-$\log_{10}$ ($P < .001$), respectively, when compared to the control groups. On the other hand, comparisons of the different SN colloidal suspensions within the same silver concentration did not indicate significant differences ($P > .05$). Thus, in general, according to the biofilm quantification results obtained in this study, the particle size and the type of SN stabilizing agent did not affect the antifungal activity of SN against *Candida* biofilms.

3.5. Discussion

Denture stomatitis is a pathogenic state common in complete denture wearers, and its infective causes include some microorganisms, mainly *C. albicans* and *C. glabrata* (Coco *et al*. 2008). Therefore, the ability of SN to inhibit the formed *Candida* biofilms was evaluated against the aforesaid microorganisms. The results of the present study did not allow the rejection of the null hypotheses, which are that the nanoparticle size and the type of stabilizing agent would not interfere with SN efficacy in decreasing total biomass and cultivable cells of *Candida* biofilms.

Since SN may not only act in the biofilm cells, but also in their matrix, CV staining assay was performed to quantify the total biomass of *Candida* biofilms. This assay does not allow differentiation between living and dead cells, so it cannot be used for susceptibility testing of biofilms. CV staining was used as a complement to CFU enumeration. From Fig. 3, it is possible to observe that the different SN were very effective against *C. glabrata* D1 biofilms, with significant biomass reduction at silver concentrations greater than or equal to 1.6 µg ml^{-1} for all conditions (diameter and solution) assayed. For this species, all SN colloidal suspensions showed biomass reductions around 90% at a silver concentration of 108 µg ml^{-1}. However, in general, for *C. albicans* 324LA/94 biofilms there was some increase in biofilm biomass when compared to untreated biofilms, including different particle sizes and solutions. These differences in the effect of SN on both species could be due to their biofilm architectures, which present several dissimilarities. *C. glabrata* biofilms are exclusively composed of blastospores and devoid of hyphal cells, while *C. albicans* is a polymorphic organism, able to grow

as hyphae, pseudohyphae, and blastospores (Silva *et al*. 2011). Accordingly, *C. albicans* biofilms are more complex and difficult to eradicate. Furthermore, the biofilm formation and matrix production processes are dependent upon species, strains and environmental conditions (Silva *et al*. 2011). When biofilms are exposed to stress conditions, physiological changes can occur to protect the cells (Mah and O'Toole 2001). This may suggest an increased production of exopolymeric substances by *C. albicans* 324LA/94 biofilms in the presence of SN, which could explain the increase in total biomass observed in Figure 3A, accompanied by a slight decrease in the number of viable biofilm cells.

Moreover, the silver concentrations above which significant reductions were detected in the number of biofilm cells of *C. albicans* 324LA/94 (Fig. 4a) and *C. glabrata* D1 (Fig. 4b), were about 17 to 135 and 16.5 to 34-fold higher, respectively, than the corresponding MICs (Table 1), confirming the lower susceptibility of biofilm cells. These MIC results revealed that all SN colloidal suspensions presented similar behaviors, all being fungicidal (in concentrations ranging from 0.4 µg ml^{-1} to 3.3 µg ml^{-1}) against the two *Candida* isolates when in the planktonic growth mode (Table 1). Additionally, these fungicidal activities were confirmed by plating the content of each well on SDA. The data obtained are in agreement with the previously published results by Hawser and Douglas (1995). These authors found that all of the antifungal agents tested (amphotericin B, flucytosine, fluconazole, itraconazole and ketoconazole) showed much less activity against 48-h *C. albicans* biofilms than against planktonic cells. The drug concentrations that caused 50% inhibition in biofilms were 30 to 2,000 times higher than the relevant MICs (Hawser and Douglas 1995). In fact, *C. albicans*

and *C. glabrata* form recalcitrant biofilms which are difficult to eradicate, (Lewis 2001) due to increasing tolerance to conventional antifungal therapy (Silva *et al*. 2012). According to Silva *et al*. (2012), the mechanisms of biofilm drug resistance/tolerance are not fully understood, but can be considered a multifactorial phenomenon. In combination or alone, these mechanisms include decreased growth rate (Lewis 2001), heterogeneity, expression of resistance genes and presence of 'persister' cells within the biofilm (Lewis 2001; Mah and O'Toole 2001). In addition, although some SN may diffuse inside the matrix through the water channels present in the biofilm architecture, the microorganisms present on the deeper layers may escape the treatment as the matrix hampers the drug diffusion (Kalishwaralal *et al*. 2010).

Regarding cell viability (Fig. 4), although all SN presented a similar behavior, they were more effective on *C. glabrata* D1 than against *C. albicans* 324LA/94 biofilms. However, for both species in general, a significant reduction in the number of biofilm viable cells for silver concentrations at or higher than 13.5 µg ml^{-1} was observed. Interestingly, the highest silver concentration (216 µg ml^{-1}) did not have a significantly greater antibiofilm effect than the lowest concentrations (54 and 108 µg ml^{-1}), characterizing a relatively weak dose-response, except for SN5-P against *C. glabrata* D1, highlighting the similarities between the particles. Accordingly, these findings also suggest that it may not be necessary to use the highest silver concentrations.

SN colloidal suspensions were prepared with NH_3 and PVP as stabilizers. NH_3 stabilizes metallic SN by the formation of soluble diammine silver (I) complexes, which trap free silver ions responsible for particle growth (Gorup *et*

al. 2011). The polymers of the PVP group bond on the SN surfaces through the nitrogen atom in their molecule (Kvítek *et al*. 2008), resulting in flocculation. Kvítek *et al*. (2008) reported that despite the flocculation process, the SN stabilized by PVP are separated from each other through the chain of the polymer molecule, and these SN can interact with the cell wall of a microorganism due to their high surface energy and mobility. Furthermore, according to the literature (Baker *et al*. 2005), smaller SN are more effective than larger particles, due to a larger surface area available for interaction with cells. However, as referred, CV staining and CFU analysis (Fig. 3 and 4) showed that the particle size range assayed and the type of stabilizing agent did not interfere with the antifungal activity of SN against *Candida* biofilms. Choi *et al*. (2010) demonstrated, through laser-scanning confocal microscopic observations, that the interactions of SN with *Escherichia coli* biofilm cells resulted in particle aggregation with a final average aggregate size of about 800 nm (an increase by a factor of 40). Moreover, Stewart and Franklin (2008) reported that biofilm features, such as oxygen availability, substrate, pH range, and extracellular polymeric substances composition, may influence aggregation, dissolution and diffusive transport of SN. Taken together, all these observations reinforce the idea that SN aggregation may have occurred and, consequently, caused an increase in particle size. Probably due to this fact, it was not possible to observe any significant difference among SN colloidal suspensions and an inverse relationship between SN size and antifungal activity.

Furthermore, it would be reasonable to hypothesize that the aggregation of SN within the biofilms led to a particle size which conflicted extensively from that originally synthesized. This fact disallowed discriminating the effect of

particle size and stabilizing agent on *Candida* biofilms. These observations emphasize that the original particle size may be a poor display of true nanoparticle size in biofilms, and therefore, also of antifungal activity. In other words, the results of the present study imply that biofilms are influencing by the aggregation of SN, and that the size of nanoparticles and the type of stabilizing agent were not crucial to their positive effect on *Candida* biofilms. Possibly, only the well dispersed SN may act against *Candida* biofilms effectively.

In summary, the particle size and the type of stabilizing agent do not interfere with the *in vitro* antimicrobial efficacy of SN. On the basis of these findings, additional investigations with silver colloidal nanoparticles will be needed regarding their therapeutic potential, namely to assess formulation and delivery means.

Acknowledgments

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Table 1. Minimum inhibitory concentrations (MICs) of silver nanoparticles (SN) stabilized with ammonia (NH_3) and polyvinylpyrrolidone (PVP) against *Candida* spp

***Candida* spp.**	**MIC (µg ml^{-1})**					
	SN stabilized with NH_3			**SN stabilized with PVP**		
	5 nm	**10 nm**	**60 nm**	**5 nm**	**10 nm**	**60 nm**
C. albicans 324LA/94	0.4-0.8	0.8	0.4	0.8	0.8-1.6	0.4-0.8
C. glabrata D1	1.6-3.3	1.6	0.8-1.6	1.6-3.3	3.3	1.6-3.3

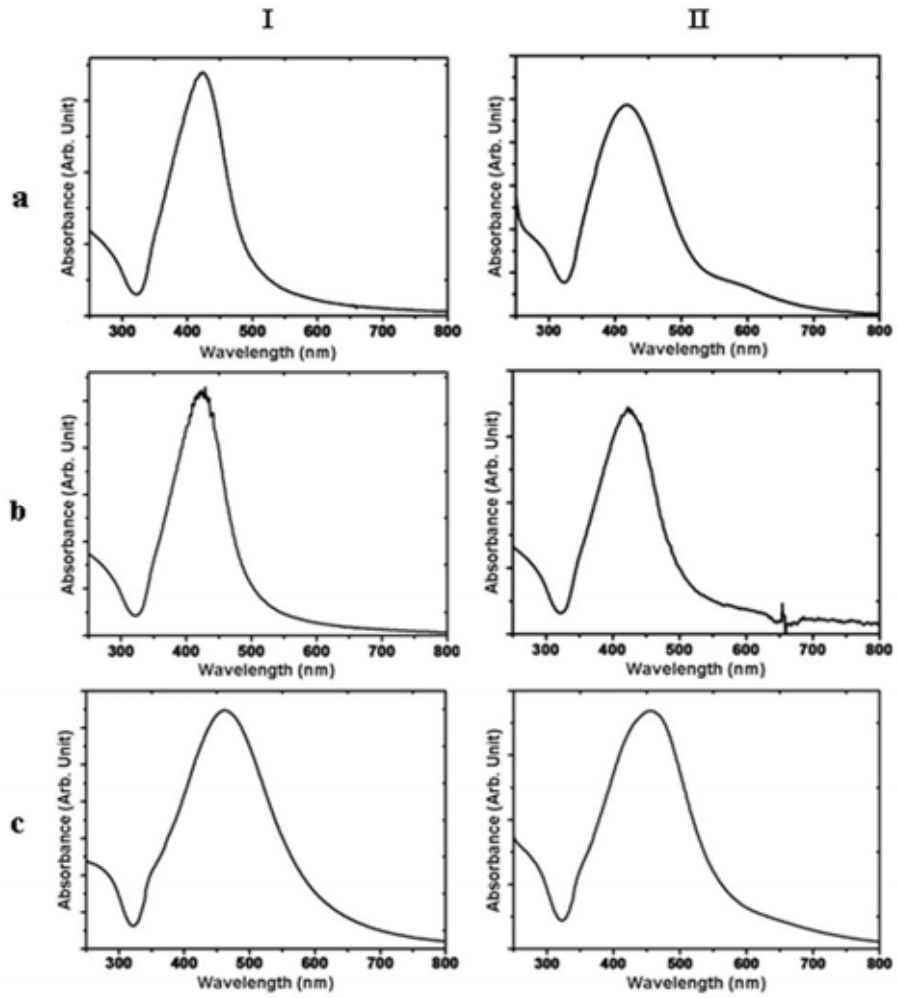

Figure 1. UV-visible spectra of silver nanoparticles (SN) colloidal suspensions with 5 (a), 10 (b) and 60 (c) nm, stabilized with ammonia (I) and polyvinylpyrrolidone (II)

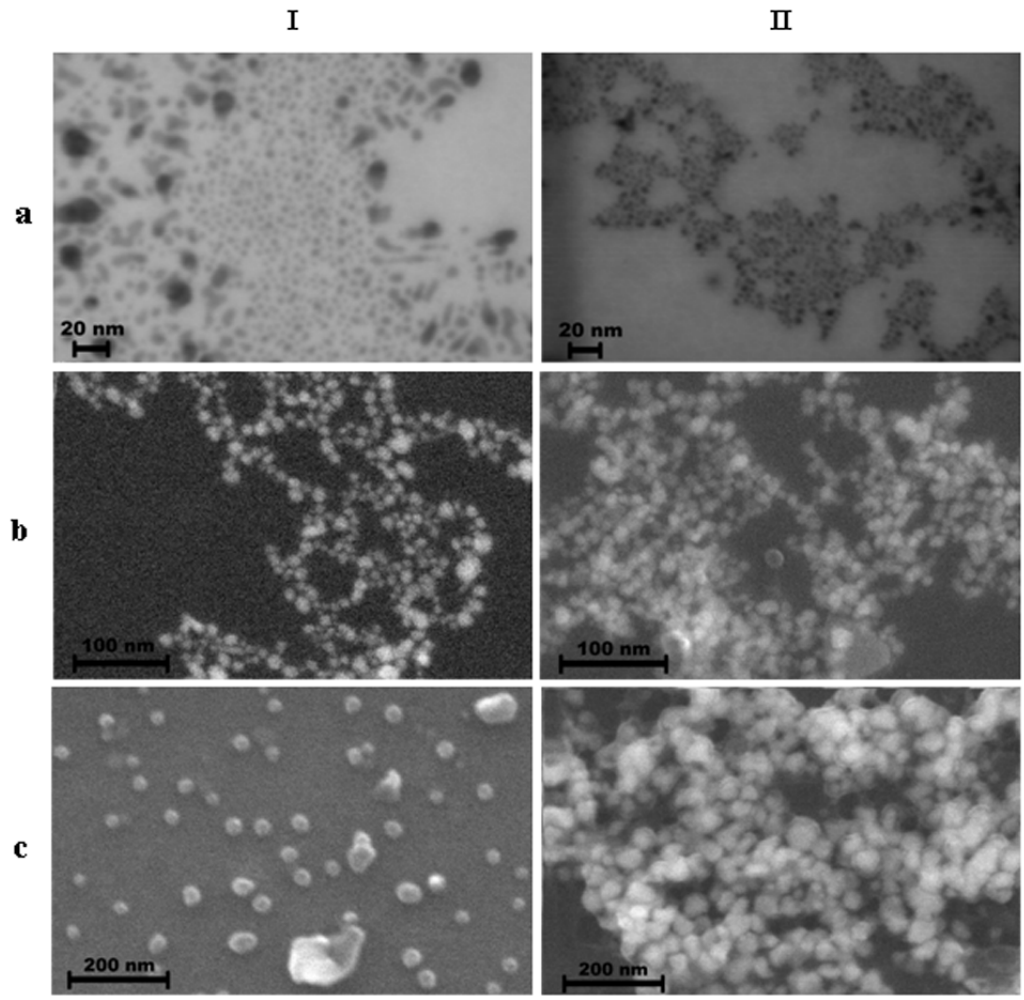

Figure 2. Transmission electron microscopy images of silver nanoparticles (SN) with 5 (a), 10 (b) and 60 (c) nm, stabilized with ammonia (I) and polyvinylpyrrolidone (II)

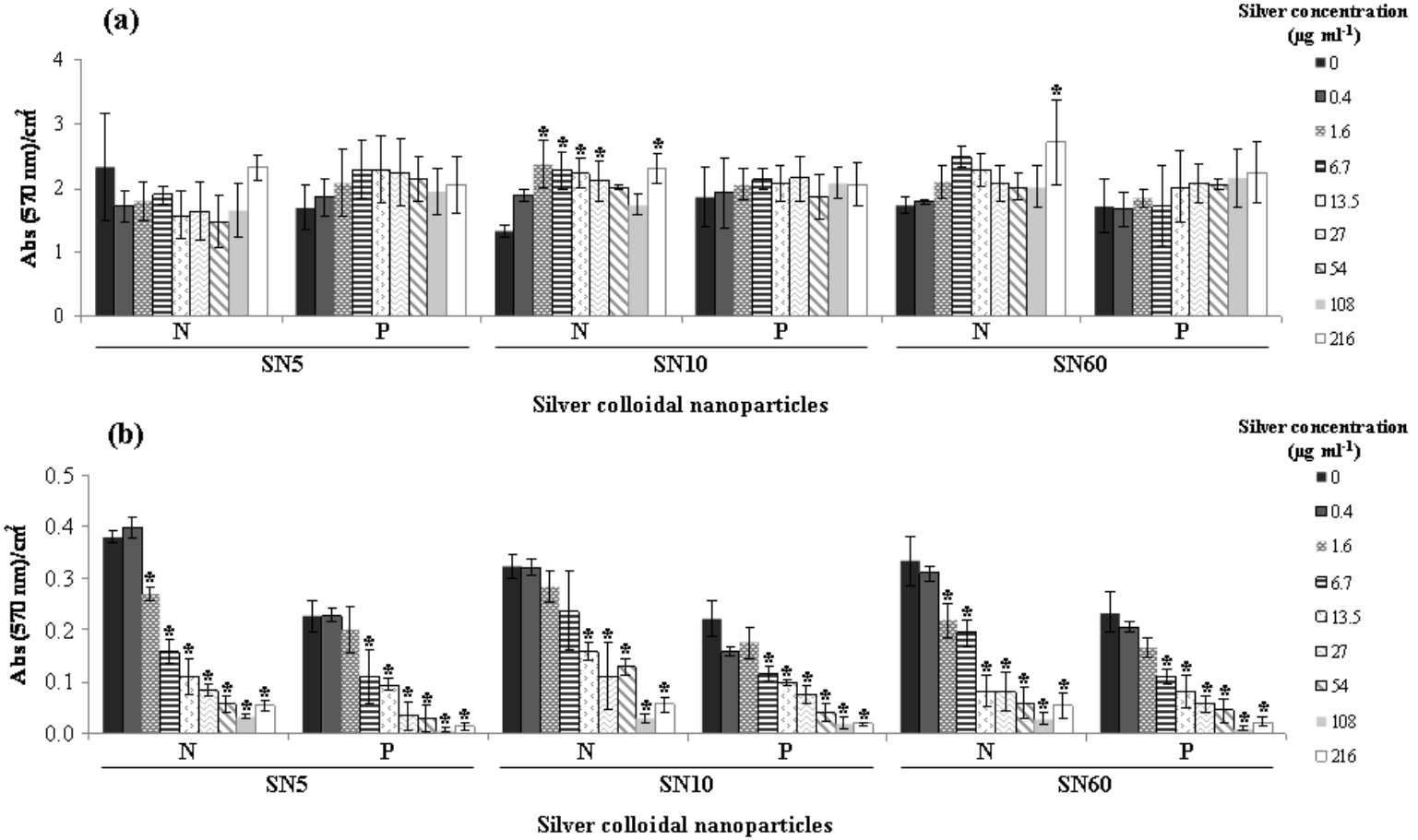

Figure 3. Absorbance values per cm^2 obtained with crystal violet (CV) staining assay for *C. albicans* 324LA/94 (a) and *C. glabrata* D1 (b) mature biofilms after 24 h of treatment with silver nanoparticles (SN) with 5 (SN5), 10 (SN10) and 60 (SN60) nm, which were stabilized using ammonia (N) and plyvinylpyrrolidone (P). Error bars indicate the standard deviations of the means. (*) Indicates $P < 0.05$, as compared to the control groups by using a one-way ANOVA followed by Bonferroni test

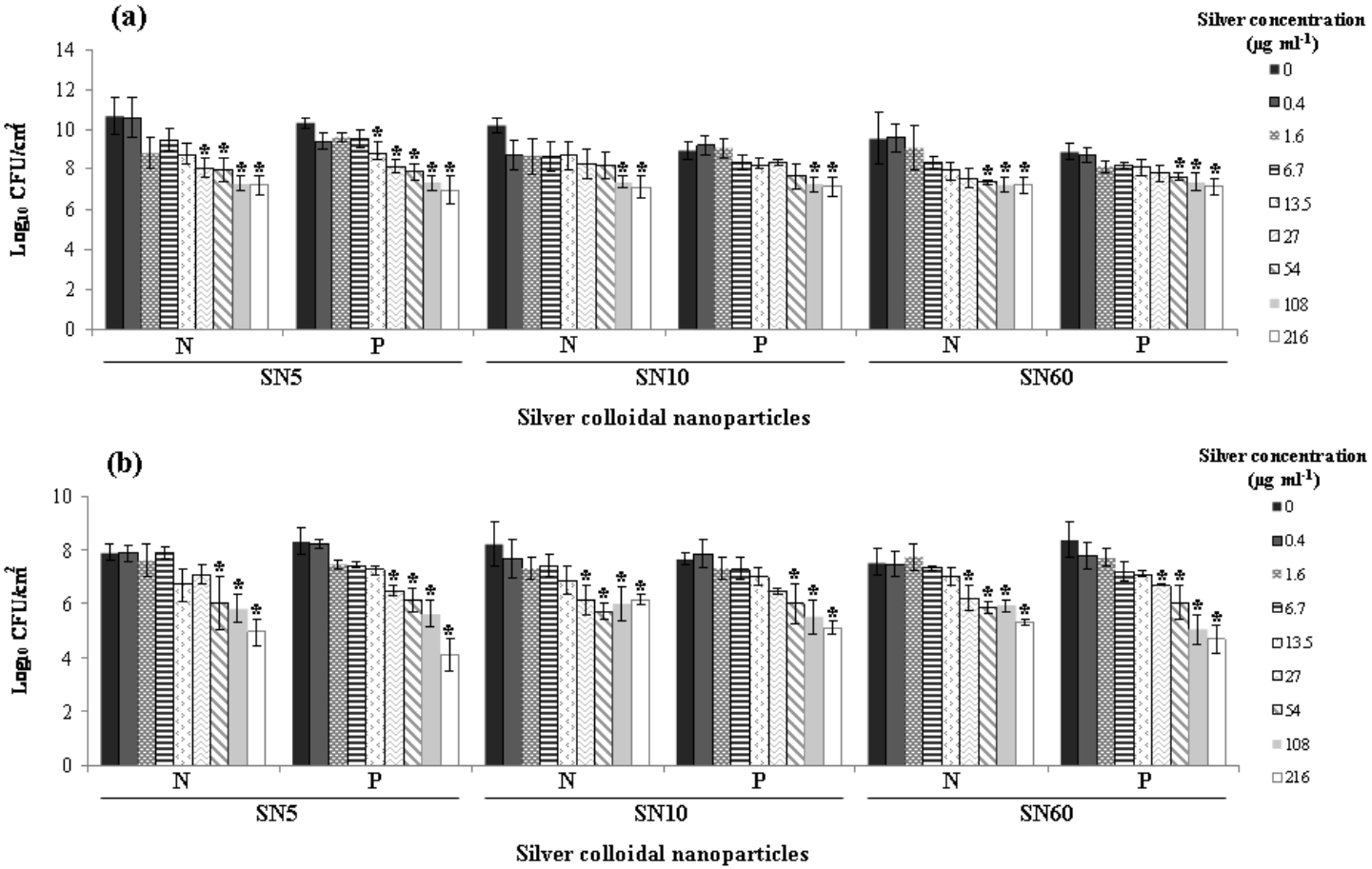

Figure 4. Logarithm of colony forming units per cm^2 obtained for *C. albicans* 324LA/94 (a) and *C. glabrata* D1 (b) mature biofilms after 24 h of treatment with silver nanoparticles (SN) with 5 (SN5), 10 (SN10) and 60 (SN60) nm, which were stabilized using ammonia (N) and plyvinylpyrrolidone (P). Error bars indicate the standard deviations of the means. (*) Denote $P < 0.05$, when each SN colloidal suspension was evaluated separately and compared to the control groups by using a one-way ANOVA followed by Bonferroni test

CAPÍTULO 3

Silver colloidal nanoparticles: effect on matrix composition and structure of *Candida albicans* and *Candida glabrata* biofilms[*]

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Silver colloidal nanoparticles: effect on matrix composition and structure of *Candida albicans and Candida glabrata* biofilms

4.1. Abstract

Aim: The aim of this study was to assess the effect of different silver nanoparticles (SN) concentrations on the matrix composition and the structure of *Candida albicans* and *Candida glabrata* biofilms. **Methods and Results:** *Candida* biofilms were developed in 6-well microtiter plates during 48 h. After, these biofilms were exposed to 13.5 or 54 µg SN ml^{-1} for 24 h. Then, extracellular matrices were extracted from biofilms and analyzed chemically in terms of proteins, carbohydrates and DNA. To investigate the biofilm structure, scanning electron microscopy (SEM) and epifluorescence microscopy were used. SN interfered with the matrix composition of *Candida* biofilms tested in terms of protein, carbohydrate and DNA, except for the protein content of *C. albicans* biofilm. By SEM, *Candida* biofilms treated with SN revealed structural differences, when compared to the control groups. Further, SN showed a trend of agglomeration within the biofilms. Epifluorescence microscopy images suggest that SN induced damage to cell walls of *Candida* isolates tested. **Conclusions:** In general, irrespective of concentration, SN affected the matrix composition and structure of *Candida* biofilms and these findings may be related to the mechanisms of biocide action of SN. **Significance and Impact of the Study:** This study reveals new insights about the behavior of SN when in contact with *Candida* biofilms. SN may contribute in the development of therapies to prevent or control *Candida* infections.

Keywords: biofilms; *Candida albicans*; *Candida glabrata*; silver nanoparticles; fungal infections.

4.2. Introduction

Candida pathogenesis is associated with biofilm formation (Vediyappan *et al*. 2010) and *Candida* spp. can colonize tissues or inert surfaces, such as oral epithelia and dental prostheses, respectively (Kojic and Darouiche 2004; Ramage *et al*. 2006). An example of oral infection caused by *Candida* biofilms is the *Candida*-associated denture stomatitis, which is characterized by the presence of biofilm in the space between the denture base and the mucosa (Budtz-Jorgensen 2000). Usually, *Candida* biofilms are resistant to host defense mechanisms and to antifungal drugs, representing a continuous source of infection (Baillie and Douglas 2000). One of the factors that may be associated with antifungal resistance of *Candida* biofilms is the presence of extracellular matrix (Mah and O'Toole 2001).

According to Flemming *et al*. (2007) the biofim matrices or extracellular polymeric substances (EPS) “are biopolymers of microbial origin in which biofilm microorganisms are embedded”. This matrix is composed of a wide variety of proteins, carbohydrates and phosphorus (Baillie and Douglas 2000; Blankenship and Mitchell 2006). In addition, extracellular DNA has also been mentioned as a component of the biofilm matrices (Martins *et al*. 2010).

The composition of extracellular material varies depending on the growth medium, environmental conditions and species (Conover *et al*. 2011). Al-Fattani and Douglas (2006) reported that *Candida albicans* biofilm matrix consisted of carbohydrate (32.2% glucose), small amounts of proteins (5.0%), hexosamine (3.3%), phosphorus (0.5%) and uronic acid (0.1%), while the matrix from

Candida tropicalis biofilms consisted mainly of hexosamine (27.4%), carbohydrates (3.3%, including 0.5% glucose), proteins (3.3%), phosphorus (0.2%) and uronic acid (1.6%). A study by Silva *et al*. (2009) demonstrated that the biofilm matrices of *Candida glabrata* had higher concentrations of proteins and carbohydrates when compared with other species (*Candida tropicalis* and *Candida parapsilosis*). On the other hand, antifungal drugs may bind to beta-glucans, which are constituents of the extracellular matrix of *Candida* biofilms, preventing the drug from reaching its target (Vediyappan *et al*. 2010). Thus, strategies that focus on eradication of matrix components may contribute to the control of infections related to biofilms.

Currently, nanotherapeutics have been used to control the biofilm formation through the incorporation of nanoparticles into several materials (Monteiro *et al*. 2009; Allaker 2010; Cheng *et al.* 2012; Monteiro *et al*. 2012a; Wady *et al.* 2012). In this context, silver nanoparticles (SN) were reported as antimicrobial agents against bacteria (Sondi and Sondi 2004) and fungi (Kim *et al*. 2009; Panácek *et al*. 2009), and their mechanism of action is multifactorial. These nanoparticles preferably bind to sulfur-containing proteins, thereby forming pores in the cell wall and membranes and leading to loss of intracellular contents. Furthermore, nanoparticles attack respiratory chain enzymes, causing cell disintegration, and interact with phosphorus in the DNA, preventing cell division (Monteiro *et al*. 2009; Rai *et al.* 2009).

According to literature data, SN were able to prevent biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Kalishwaralal *et al.* 2010). Encouraging results about the effect of SN against *C. albicans* and *C.*

glabrata adhered cells and biofilms were also reported (Monteiro *et al*. 2011; Monteiro *et al*. 2012b). However, there are no studies on the effect of these nanoparticles in the matrix composition and structure of *Candida* biofilms. Therefore, the main aim of this study was to evaluate the effect of different SN concentrations on the matrix composition (protein, carbohydrate and DNA content) and the structure (using Scanning Electron Microscopy (SEM) and Epifluorescence Microscopy) of *C. albicans* and *C. glabrata* biofilms.

4.3. Materials and methods

Preparation of silver colloidal nanoparticles

For the preparation of silver colloidal nanoparticles all chemicals were obtained from Merck KGaA, Darmstadt, Hesse, Germany. These nanoparticles were synthesized by a process that involves reduction of silver nitrate ($AgNO_3$) with sodium citrate ($Na_3C_6H_5O_7$) and their stabilization with ammonia (NH_3), as evidenced earlier (Gorup *et al*. 2011; Monteiro *et al*. 2011; Monteiro *et al*. 2012a; Monteiro *et al.* 2012b). Briefly, a solution of $AgNO_3$ (5.0×10^{-3} mol l^{-1}) in deionized water was brought to boiling and an aqueous solution of $Na_3C_6H_5O_7$ was added to the $AgNO_3$ solution. The solution turned yellow and a colloidal suspension was formed. Next, the silver colloids were stabilized by adding of 7.5 ml of an ammonia solution (1.4 mol l^{-1}). The silver colloidal nanoparticles suspensions were characterized as detailed in our previous studies (Gorup *et al*. 2011; Monteiro *et al*. 2011, Monteiro *et al*. 2012a; Monteiro *et al.* 2012b), and SN having an average particle size of 5 nm were used.

Microorganisms and preparation of *Candida* cell suspension

The composition (per 1 l of deionized water) of artificial saliva medium (Lamfon *et al*. 2003) used in this study was: 2 g of yeast extract (Liofilchem, Roseto degli Abruzzi, Italy), 5 g of peptone (Liofilchem), 2 g of glucose (AppliChem, Darmstadt, Germany), 1 g of mucin (Sigma-Aldrich, St Louis, USA), 0.35 g of

NaCl (AppliChem), 0.2 g of $CaCl_2$ (Riedel-de Haën, Seelze, Germany) and 0.2 g of KCl (Pronalab, Lisbon, Portugal). The pH was adjusted to 6.8.

An oral clinical isolate (324LA/94) of *C. albicans*, provided by the culture collection of Cardiff Dental School, Cardiff, UK, and an oral clinical isolate (D1) of *C. glabrata*, obtained from the biofilm group of the Centre of Biological Engineering, University of Minho, Braga, Portugal, were used in all of the experiments. All *Candida* strains were stored as frozen stocks with 20% glycerol at -80ºC until required. To prepare the *Candida* cell suspension for biofilm growth, clinical isolates were subcultured on Sabouraud dextrose agar (SDA, Liofilchem) plates for 24 h at 37º C and then, a loopful of the *Candida* colonies was transferred into 30 ml of Sabouraud dextrose broth (Liofilchem) and incubated overnight at 37ºC in a rotary shaker (at 120 rev min^{-1}). Afterwards, *Candida* cells were harvested by centrifugation (6500 g for 5 min at 15ºC), washed twice with 30 ml of phosphate buffered saline (PBS; pH 7), enumerated using a Neubauer chamber and then adjusted to a concentration of 10^7 cells ml^{-1} in artificial saliva medium. These *Candida* cell suspensions were used in the subsequent assays.

Biofilm formation and treatment with SN

Biofilm formation was performed as described by Silva *et al*. (2009). Briefly, 4 ml of standard cell suspensions of yeasts prepared as above were added into each well of a 6-well polystyrene microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium), and the plate incubated with shaking (120 rev min^{-1}) for 48 h at 37ºC.

After 24 h, an aliquot of 2 ml of artificial saliva medium was removed and an equal volume of fresh artificial saliva was added. After the incubation period, non-adherent cells were removed by washing with 4 ml of PBS.

Stock suspensions of silver colloidal nanoparticles were diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) to obtain two different concentrations of silver: 54 and 13.5 μg ml^{-1}. These SN concentrations are representative of the maximum and intermediate concentrations tested in a previous study (Monteiro *et al.* 2011). A volume of 4 ml of each dilution was transferred to the wells of the 6-well polystyrene microtiter plate, containing pre-formed biofilms. In control experiments, RPMI 1640 medium without SN was added to the wells. The plates were incubated at 37ºC for 24 h with shaking (120 rev min^{-1}). After treatment with SN, biofilms were washed once with 4 ml of PBS to remove loosely attached cells.

Biofilm matrix extraction

After treatment with SN, the biofilm matrix was extracted in accordance with a previously described procedure (Silva *et al*. 2009). Biofilms were scraped from the wells of 6-well microtiter plates using sterilized cell scrapers (Orange Scientific) and resuspended with PBS. Following, the biofilm samples were sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30s at 30 W and the suspension was vortexed for 2 minutes. Finally, the suspension was centrifuged at 3000 g for 10 min at 4ºC and the supernatant filtered through a 0.45 μm nitrocellulose filter (Orange Scientific).

Biofilm dry weight determination

For this assay, biofilms removed from the 6-well plates were filtered on 0.45 µm sterilized membrane (Pall Corporation, Michigan, USA) and dried at 60ºC until a constant dry weight. The membrane was also weighed before biofilm filtering. Thus, biofilm dry weight was obtained as the difference between the 2 measurements.

Protein, carbohydrate and DNA quantification

The protein content was determined using the BCA Kit (Bicinchoninic Acid, Sigma-Aldrich), using bovine serum albumin (BSA) as the standard (Silva *et al*. 2009). Briefly, 25 µl of the suspension obtained after biofilm matrix extraction were transferred into wells of 96-well microtiter plates containing 200 µl of the mixture of BCA kit reagents. The plates were incubated at 37ºC for 30 minutes and the absorbance read in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 562 nm.

The total carbohydrate content was measured according to the procedure of Dubois *et al*. (1956), using glucose as a standard. Thus, into a glass tube, 500 µl of the suspension obtained after biofilm matrix extraction were mixed with 500 µl of 9% phenol (Panreac, Barcelona, Spain) and 2.5 ml of sulfuric acid (95-97%, Applichem). The solution was homogenized by vortexing and after 15 minutes at room temperature the absorbance was read at 490 nm.

To assess the DNA content in biofilm matrix, a small volume (1.5 µl) of

the suspension obtained after biofilm matrix extraction was spectrophotometrically analyzed (at 260 nm and 280 nm) using a Nanodrop Spectrophotometer (Thermo Scientific, Nanodrop 1000, USA).

Total proteins, carbohydrates and DNA contents were expressed as a function of the biofilm dry weight (mg g^{-1} of biofilm dry weight). Three to six separate experiments were performed for each condition studied.

Biomass and cultivable cells quantification

Biofilm biomass, obtained after treatment with silver colloidal nanoparticles, was evaluated by the crystal violet (CV) staining method, as previously described (Silva *et al*. 2009; Monteiro *et al*. 2011). Briefly, biofilms were fixed with 4 ml of 99% methanol (Romil, Cambridge, UK). After 15 minutes, the methanol was removed and the microtiter plates were allowed to air-dry. *Candida* biofilms were stained with 4 ml of CV stain (1% v v^{-1}) (Merck) for 5 minutes. The plates were washed with deionized water, allowed to dry at room temperature, and then the CV was removed by adding 4 ml of acetic acid (33% v v^{-1}) (Pronalab) into each well. Afterwards, 200 µl of the solution were transferred to a 96-well microtiter plate and the absorbance was measured at 570 nm. The experiments were performed in triplicate and on three separate occasions.

In addition, biofilms exposed to SN were scraped from the microtiter plate wells and 100 µl samples were taken to culturability assessment. Appropriate dilutions of biofilm cell suspension were plated on SDA, in triplicate. The SDA plates were incubated at 37ºC, and after 24 h the number of colony forming units

(CFU) per unit area of wells was enumerated. At least three independent assays were performed.

Biofilm structure

Candida biofilm structure was visualized by SEM. First, *Candida* biofilms were developed within the 24-well microtiter plates by dispensing standardized yeasts cell suspensions (1 ml of a suspension containing 10^7 cells ml^{-1} in artificial saliva) into each well and incubated for 48 h at 37ºC in a shaker at 120 rev min^{-1}. After treatment with different SN concentrations (54 and 13.5 µg ml^{-1}) for 24 h, the RPMI 1640 medium was aspirated and each well was rinsed gently with PBS. Subsequently, samples were dehydrated by washing in a series of ethanol (70% for 10 min, 95% for 10 min and 100% for 20 min), and finally air dried in a desiccator. Afterwards, samples were mounted onto aluminum stubs, sputter coated with gold and viewed under a S-360 scanning electron microscope (Leo, Cambridge, USA).

Biofilm cells structure was also observed by Epifluorescence Microscopy. After treatment with SN, biofilm cells were fixed overnight at room temperature and stained with Calcofluor (Calcofluor White Stain, Sigma-Aldrich) (diluted 1:100 in ultra pure water) during 15 min. Prior to the microscopy observation, the cells were thoroughly washed with deionized water and observed using an Epifluorescence Microscope (BX51; Olympus, Tokyo, Japan) equipped with a CCD camera (DP71; Olympus) and filter capable of detecting the yeast cell wall (BP 365–370, FT 400, LP 421).

Statistical analysis

The results of triplicate experiments conducted for each of the above assays were analyzed applying ANOVA followed by Bonferroni multiple comparison post-hoc test, using SPSS software (SPSS - Statistical Package for the Social Sciences, Inc., Chicago). $P < 0.05$ was considered statistically significant.

4.4. Results

Protein, carbohydrate and DNA quantification

Table 1 summarizes the results of quantitative analysis of total proteins, carbohydrates and DNA extracted from *Candida* biofilm matrices after treatment with different SN concentrations. For *C. albicans* 324LA/94, the protein content of biofilm matrices was completely unaffected by SN treatment (P = 0.935, ANOVA). On the other hand, the treatment with 13.5 and 54 µg SN ml^{-1} for 24 h increased the total carbohydrate content, but this increase, when compared to the control group, was only significant (~71%, P = 0.021) for the SN concentration of 54 µg ml^{-1}. There was a significant reduction in *C. albicans* 324LA/94 biofilm DNA content when treated with 13.5 µg SN ml^{-1} compared to the control group (~36%, P = 0.002). However, there was no significant difference in the DNA content between the control group and the biofilm treated with 54 µg SN ml^{-1}.

For *C. glabrata* D1, the matrix of biofilm treated with 13.5 µg SN ml^{-1} had a higher amount of proteins and DNA than the control group (P = 0.001; P = 0.038) and the group treated with 54 µg SN ml^{-1} (P < 0.001; P = 0.001). It was also observed that the matrix of biofilm treated with 54 µg SN ml^{-1} contained less total proteins and DNA compared to the control group, but this decrease was only significant for DNA content (~ 33%, P = 0.011). In addition, biofilm matrix of *C. glabrata* D1 treated with 13.5 µg SN ml^{-1} contained less total carbohydrates than the control group. However, interestingly, after treatment with 54 µg SN ml^{-1} occurred significant increase regarding the total carbohydrates content compared

to the control group (~158%, $P < 0.001$) and to the group treated with 13.5 µg SN ml^{-1} (~270%, $P < 0.001$).

Biomass and cultivable cells quantification

The efficacy of SN colloidal suspensions in reducing total biomass and the number of CFUs of *Candida* biofilms is shown in Fig. 1. According to Fig. 1(a), when compared to the control groups (biofilms without SN), *C. albicans* 324LA/94 and *C. glabrata* D1 achieved biofilm biomass reductions around 50% ($P < 0.001$) and 47% ($P = 0.033$) at a silver concentration of 13.5 µg ml^{-1} and around 54% ($P < 0.001$) and 90% ($P = 0.001$) at a concentration of 54 µg ml^{-1}, respectively.

With regard to biofilm cultivable cells (Fig. 1b), it was possible to observe that the treatment (during 24 h) with SN colloidal suspensions decreased the number of CFUs for *C. albicans* 324LA/94, however only the concentration of 54 µg ml^{-1} showed a significant statistical decrease (reduction of 2.12-log_{10}; $P = 0.010$) when compared to the control group. For *C. glabrata* D1 biofilms, the effect of SN was more pronounced, with decreases of 3.86-log_{10} ($P = 0.001$) and 6.46-log_{10} ($P < 0.001$) at silver concentrations of 13.5 and 54 µg ml^{-1}, respectively. Furthermore, for *C. glabrata* D1 biofilms, the reduction in total biomass (Fig. 1a) and in the number of CFUs (Fig. 1b) were dependent on silver concentration and there were statistically significant differences ($P = 0.026$ and $P = 0.009$, respectively) between the silver concentrations tested.

Biofilm structure

Biofilm structure was monitored by SEM (Fig. 2). In the control (Fig. 2ia), *C. albicans* 324LA/94 biofilm consisted of a mixture of yeasts and hyphae surrounded by a thick extracellular matrix. The treatment with 13.5 µg SN ml^{-1} (Fig. 2ib) did not have any visible effect on the structure of *C. albicans* 324LA/94 biofilm, and similarly to that observed in Fig. 2(ia), this biofilm synthesized large amounts of extracellular matrix material and the fungal cells were almost hidden by the matrix. By contrast, the treatment of *C. albicans* 324LA/94 biofilm with 54 µg SN ml^{-1} (Fig. 2ic) resulted in a much less compact biofilm and lower amounts of extracellular polymeric material, when compared to the control group.

Interestingly, the structure of *C. glabrata* D1 biofilms was affected by SN. In the control group (Fig. 2iia), this strain revealed a multilayer biofilm composed entirely of yeasts. However, after treatment with SN in both concentrations of 13.5 (Fig. 2iib) and 54 µg ml^{-1} (Fig. 2iic), the biofilms displayed a more compact structure than the control group and having a thinner layer of yeasts covering the surface. Additionally, it was possible to observe some clusters of SN (Figs. 2ib, 2ic, 2iib and 2iic) attached to the *Candida* biofilms matrices and to the fungal cells.

Epifluorescence microscopy was also used to screen for structural differences between the biofilm cells in the absence or presence of SN. The fluorescent dye Calcofluor white used in this assay binds to the fungal cell wall allowing its visualization. As observed by SEM, mature *Candida* biofilms, under different experimental conditions (without SN and after treatment with SN)

consisted of a network of cells of all morphologies: yeasts and filamentous forms (pseudohyphae and hyphae) for *C. albicans* 324LA/94 biofilms and only yeasts for *C. glabrata* D1 biofilms, as shown in Fig. 3. Moreover, in general, increasing the silver concentration, the amount of fluorescence and cell walls stained with Calcofluor white decreased, for both biofilms (Fig. 3), suggesting that SN induced damage to cell walls of *Candida* biofilms.

4.5. Discussion

In the study described here, *C. glabrata* D1 biofilm matrix treated with SN showed significant modifications in the protein and DNA contents compared to untreated biofilm. At a silver concentration of 13.5 µg ml^{-1}, it was possible to observe statistically significant increases in the protein and DNA contents of *C. glabrata* D1 biofilm matrix, when compared to the control group (Table 1), probably due to fungal cell lysis and consequent release of intracellular contents. However, the treatment with the highest SN concentration (54 µg ml^{-1}) showed a significant reduction in the matrix protein content compared with the group treated with 13.5 µg SN ml^{-1}, and a significant reduction in DNA content, when compared to the control group (Table 1). As silver has affinity for proteins (Furno *et al*. 2004) and for phosphate groups present in the DNA molecule (Rai *et al.* 2009), one reason for the results obtained may be the dissolution of the protein and DNA contents of extracellular matrix during its exposure to SN at high concentration. Thus, SN at 54 µg ml^{-1} were able to promote cell lysis, release of intracellular constituents, and dissolution of a part of released protein and DNA.

Surprisingly, the phenomena described above were not observed for *C. albicans* 324LA/94. The total protein content of this biofilm matrix was not affected by treatment with SN. In addition, *C. albicans* 324LA/94 biofilm exposed to 13.5 µg SN ml^{-1} demonstrated a significant decrease in DNA content compared to the control, while the group treated with SN at 54 µg ml^{-1} did not differ from the control. DNA can be released into extracellular matrix by dead cells (Mulcahy *et al.* 2008), from DNA containing outer membrane vesicles (Schooling and

Beveridge 2006), or can be regulated by quorum sensing (Allesen-Holm *et al*. 2006). As *C. albicans* biofilms are more complex than those of *C. glabrata*, the results obtained for protein and DNA contents of *C. albicans* 324LA/94 biofilm might result from the increased or decreased expression of genes involved, for example, in amino acid and phosphate biosyntheses, in response to SN exposure. In this regard, Vediyappan *et al*. (2010) found that *C. albicans* biofilms exposed to caspofungin exhibited marked changes in the expression of hypha-specific genes (such as ALS3 and HWP1), irrespective of the antifungal concentration used.

The major components of EPS are carbohydrates (Al-Fattani and Douglas 2006; Paramonova *et al*. 2009). Moreover, some studies have shown that the cell wall of *C. albicans* is composed by 80 to 90% of carbohydrates, and the most frequently found are β-glucans (Nett *et al*. 2007; Paramonova *et al*. 2009). The fungal cell wall provides physical strength, limits permeability, and their components may contribute for resistance to antifungal agents (de Groot *et al*. 2008).

In general, after treatment with 54 μg SN ml^{-1}, both *C. albicans* 324LA/94 and *C. glabrata* D1 demonstrated significant increases in total carbohydrate content compared to the untreated biofilm groups (Table 1). The increase in carbohydrate content was higher for *C. glabrata* D1 biofilm, on which SN were more effective in reducing total biomass and number of CFUs (Fig. 1). These results should be seen in conjunction with the epifluorescence microscopy assay, which showed that SN induced damage to cell walls of both *Candida* biofilms, mainly at silver concentration of 54 μg ml^{-1} (Figs. 3ic and 3iic). Thus, taken

together, these results suggest that the increases in carbohydrate content can be associated with changes in cell walls and secretion of their carbohydrates.

Furthermore, it is perhaps not surprising the apparent increase in the amount of extracellular matrix of *C. glabrata* D1 biofilm, compared to the control group, seen in Fig. 2(iic). During treatment with SN, in addition to changes in cell walls, the surviving cells could increase the production of exopolymeric substances in an attempt to protect the biofilm, and this fact may have contributed to the increase in carbohydrate content observed. However, as the Fig. 2 (ic) shows a apparent decrease in the amount of EPS, the increase in carbohydrate content for *C. albicans* 324LA/94 biofilm treated with SN at 54 µg ml^{-1} (Table 1) may represent only the carbohydrates that were released from cell walls affected by SN.

Parallel experiments performed to examine the effects of SN on total biomass and number of CFUs (Fig. 1a and 1b) showed that SN were more effective in eradicating *C. glabrata* D1 biofilms, achieving a total biomass reduction around 90% at a silver concentration of 54 µg ml^{-1}, and reductions in the number of biofilm cells of 3.86-log_{10} and 6.46-log_{10} at silver concentrations of 13.5 and 54 µg ml^{-1}, respectively. These findings confirm those reported in a previous study (Monteiro *et al*. 2011), which also revealed the greater susceptibility of *C. glabrata* biofilms to SN compared to *C. albicans* bofilms. However, the same SN concentrations tested in the present study (13.5 and 54 µg ml^{-1}) were less effective against *C. albicans* and *C. glabrata* biofilms developed in the 96-well microtiter plates (Monteiro *et al*. 2011), especially regarding to total biomass of *C. albicans* 324LA/94. Probably, the higher shear force acting on the

biofilms formed in 6-well plates, compared with the force on the biofilms in 96-well plates, may have facilitated the diffusive transport of SN into and out of the biofilms (Stewart 2012), contributing to a better antifungal action of these nanoparticles.

On the other hand, SEM observations described here (Fig. 2) revealed a trend of SN aggregation, which was most obvious at the highest silver concentration (54 µg ml^{-1}). These findings are in agreement with those of Choi *et al*. (2010), who observed SN aggregation in the presence of *Escherichia coli* biofilms, resulting in an increase of total particle size. It seems reasonable to speculate that SN might bind to EPS, which have polyanionic nature (Hoyle *et al*. 1990), thus accumulating in the biofilm and retarding their diffusion (Hoyle *et al*. 1990). Additionally, these observations support the conclusion that the original particle size may be a poor exhibit of true nanoparticle size in biofilms (Monteiro *et al.* 2012b). Possibly, SN agglomerated were unable to kill biofilm cells completely because these particles did not reach their targets.

In summary, it may be concluded that irrespective of concentration, SN affected the matrix composition of *Candida* biofilms assessed in terms of proteins, carbohydrates and DNA, except for the protein content of *C. albicans* 324LA/94 biofilm, which remained unchanged. After treatment with SN, SEM observations revealed structural differences for both biofilms with regard to spatial arrangement, and it was possible to observe clusters of SN on the biofilms. Moreover, epifluorescence microscopy images show that SN induced damage to the cell walls of the *Candida* oral isolates tested, especially at the highest silver concentration assayed (54 µg ml^{-1}). Finally, a limitation of this study is that only

one strain of each *Candida* species was evaluated. Furthermore, future works are needed to fully elucidate how SN can act within *Candida* biofilms. Researches focusing on this issue are crucial for the development of alternative therapies that may prevent or control *Candida*-associated denture stomatitis and other *Candida* infections.

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Table 1. Mean results and standard deviation for the amounts of each matrix component of *Candida* biofilms obtained after treatment with two different silver nanoparticles concentrations

Matrix composition (mg g^{-1} of biofilm dry weight)	***C. albicans 324LA/94***			***C. glabrata D1***		
	Silver concentration (µg ml^{-1})			**Silver concentration (µg ml^{-1})**		
	0	**13.5**	**54**	**0**	**13.5**	**54**
Proteins	27.28 ± 6.34	27.37 ± 3.25	28.56 ± 4.09	8.67 ± 0.40	27.93 ± 5.41*	0.34 ± 0.02
Carbohydrates	202.33 ± 52.05	222.64 ± 30.24	346.77 ± 47.50*	238.94 ± 73.72	166.84 ± 42.36	617.88 ± 37.86*
DNA	15.02 ± 1.38	9.66 ± 0.38*	14.20 ± 1.01	10.07 ± 1.27	12.60 ± 0.66*	6.73 ± 0.54*

*Indicates $P < 0.05$, as compared to the control group, using ANOVA with Bonferroni post-hoc test

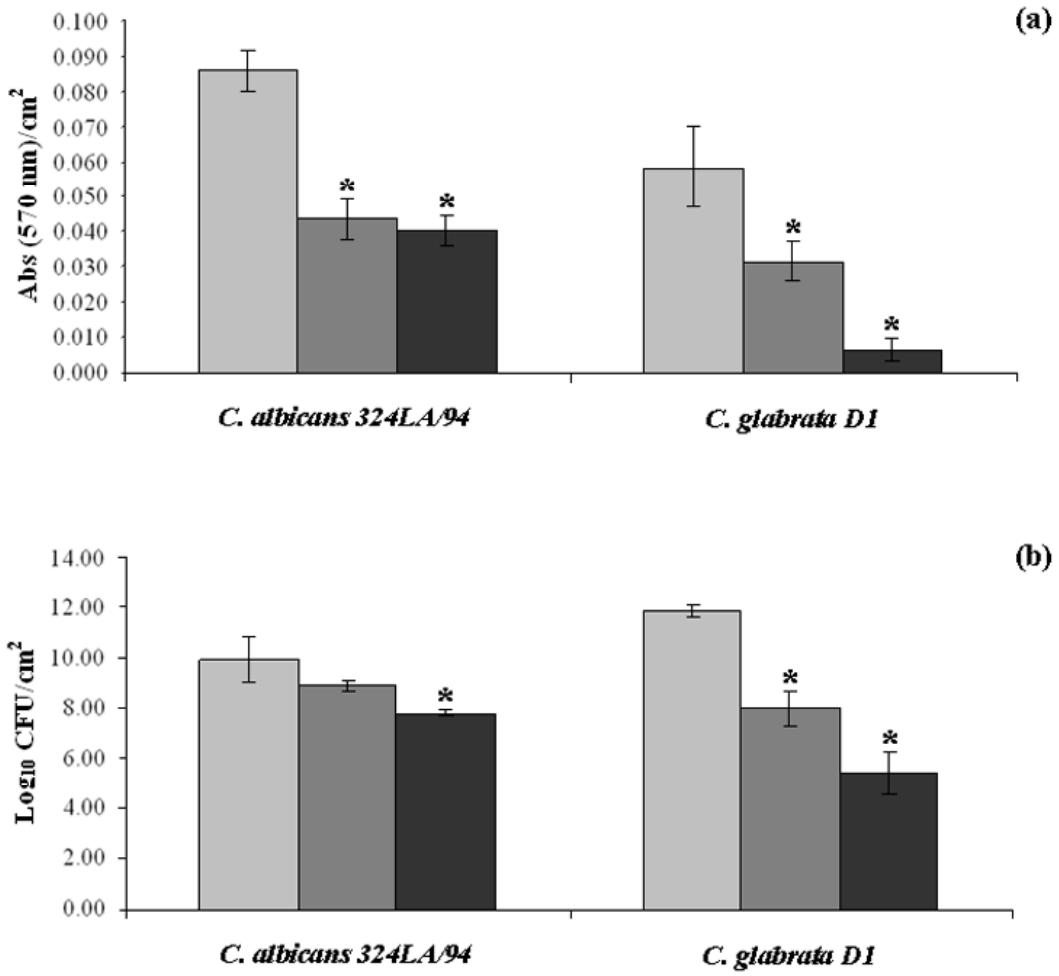

Figure 1. Absorbance values per cm^2 (a) obtained with crystal violet staining assay and logarithm of colony forming units per cm^2 (b) for *Candida albicans* 324LA/94 and *Candida glabrata* D1 biofilms treated with two different silver nanoparticles concentrations. Error bars denote the standard deviations of the means. $^{*}P < 0.05$, as compared to the control groups by using ANOVA with Bonferroni post-hoc test. Silver concentration (µg ml^{-1}): (□) 0; (▩) 13.5 and(■) 54

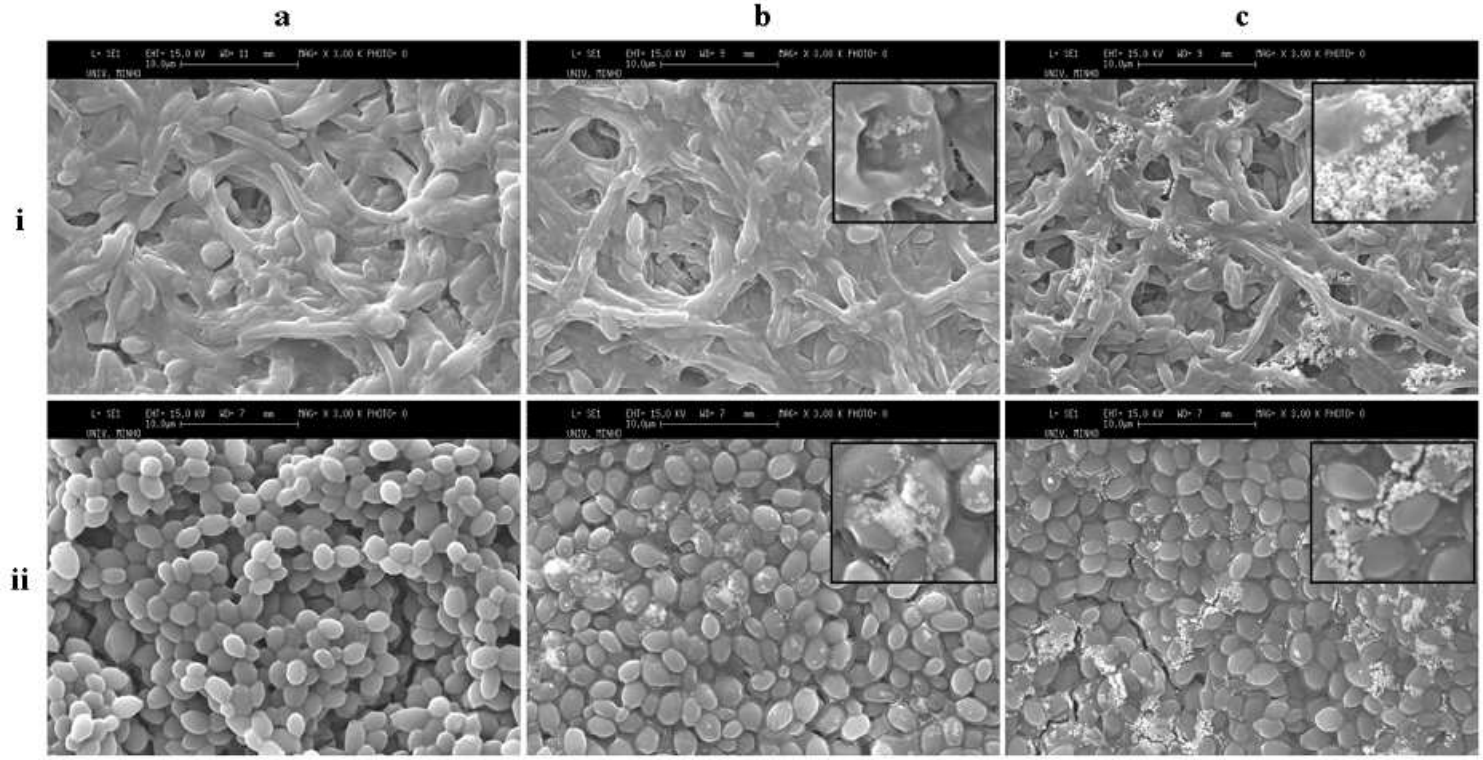

Fig. 2. Scanning electron microscopy images showing the structure of *Candida albicans* 324LA/94 (i) and *Candida glabrata* D1 (ii) mature biofilms (48 h) under different experimental conditions: (a) control group, (b) experimental group (mature biofilms treated during 24 h with silver nanoparticles at 13.5 μg ml^{-1}) and (c) experimental group (mature biofilms treated with silver nanoparticles at 54 μg ml^{-1}). Note agglomerated silver nanoparticles in an enlarged view of part of biofilms (images ib, ic, iib and iic)

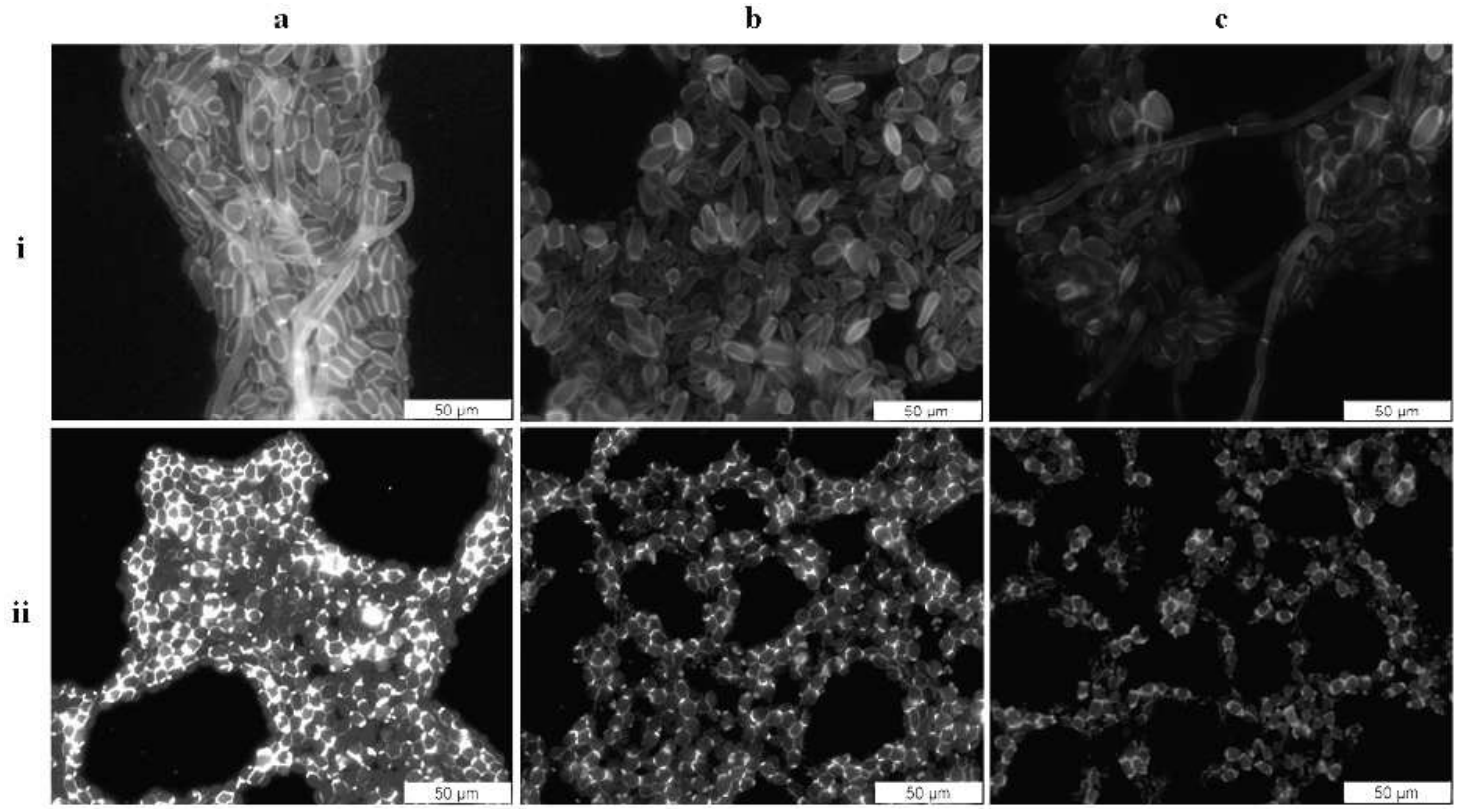

Figure 3. Epifluorescence microscopy images showing the structure of *Candida albicans* 324LA/94 (i) and *Candida glabrata* D1 (ii) mature biofilms (48 h) under different experimental conditions: (a) control group, (b) experimental group (mature biofilms treated during 24 h with silver nanoparticles at 13.5 µg ml^{-1}) and (c) experimental group (mature biofilms treated with silver nanoparticles at 54 µg ml^{-1})

CAPÍTULO 4

Antifungal activity of silver nanoparticles in combination with nystatin and chlorhexidine digluconate against *Candida albicans* and *Candida glabrata* biofilms[*]

[*]*Artigo enviado para publicação no periódico Journal of Pharmacy and Pharmacology*

Antifungal activity of silver nanoparticles in combination with nystatin and chlorhexidine digluconate against *Candida albicans* and *Candida glabrata* biofilms

5.1. Abstract

Objective: Although silver nanoparticles (SN) have been investigated as an alternative to conventional antifungal drugs in the control of *Candida*-associated denture stomatitis, the antifungal activity of SN in combination with antifungal drugs against *Candida* biofilms remains unknown. Therefore, the aim of this study was to evaluate the antifungal efficacy of SN in combination with nystatin (NYT) or chlorhexidine digluconate (CHG) against *Candida albicans* and *Candida glabrata* biofilms. ***Methods:*** The drugs alone or each combined with SN were applied on mature *Candida* biofilms (48 h), and after 24 h of treatment their antibiofilm activities were assessed by total biomass quantification (by crystal violet (CV) staining) and colony forming units (CFU) enumeration. The structure of *Candida* biofilms was analyzed by scanning electron microscopy (SEM) images. ***Key findings:*** The data indicated that SN combined with either NYT or CHG demonstrated synergistic antibiofilm activity, and this activity was dependent on the species and the drug concentrations used. SEM images showed that some drug combinations were able to disrupt *Candida* biofilms. ***Conclusion:*** The results of this study suggest that the combination of SN with NYT or CHG may have an important role in the treatment of denture stomatitis. However, further studies are needed to use these drugs safely.

Keywords: silver nanoparticles, nystatin, chlorhexidine digluconate, synergism, *Candida* biofilms

5.2. Introduction

It is well known that *Candida* species in biofilms, either on oral epithelium[1] and dentures,[2] display tolerance/resistance to antifungal drugs,[2,3] contributing to increase the risk of oral candidosis like *Candida*-associated denture stomatitis. *Candida albicans* followed by *Candida glabrata* have been identified as the most prevalent microorganisms found in denture stomatitis.[4] Several antifungal drugs are available for the management of oral yeast infections. Nystatin (NYT) (a polyene antifungal) is widely prescribed, and due to its systemic toxicity is used topically or in oral suspensions.[5] Furthermore, chlorhexidine digluconate (CHG), as a 0.2% mouthwash, has also been recommended in the treatment of oral candidosis, but its use over a long period of time can lead to tooth staining and bitter taste .[6]

The activity of antifungal combinations against *Candida* spp. has previously been described. In the study of Barkvoll and Attramadal[7] all combinations of NYT and CHG did not show efficacy against *C. albicans*. Moreover, Tobudic et al.[8] found that the combination amphotericin B/posaconazole yielded synergism, whereas Miceli et al.[9] observed that doxycycline in combination with low concentrations of amphotericin B had an antagonistic effect against several *Candida* isolates.

Over the last decade, silver nanoparticles (SN) have been intensively investigated because of their antimicrobial properties.[10] Nanoparticles have a smaller size, larger ratio of surface area per volume and higher surface area available for contact with microorganisms than microparticles.[10] SN bind to sulfur-containing proteins in biological molecules resulting in defects in the

microbial cell membrane and loss of intracellular contents. Inside the cells, the nanoparticles inhibit respiratory chain enzymes, leading to death of the microbial cells. Moreover, SN can bind to phosphorus containing compounds like DNA, preventing cell reproduction.[10,11] For instance, regarding dental applications, these nanoparticles were incorporated in acrylic resin for prevention of denture stomatitis.[12] Interestingly, our previous works attested that SN were more effective in inhibiting *Candida* biofilm formation than in controlling mature biofilms[13], and the size of nanoparticle and the type of stabilizing agent were not crucial to their efficacy against *Candida* biofilms.[14] However, a concentration-dependent toxicity has been documented for SN.[15]

Considering the aforesaid context, the use of SN in combination with conventional antifungal drugs at low concentrations against *Candida* biofilms would be desirable. Hence, the aim of the current study was to evaluate, through the quantification of total biomass and cultivable cells, the antifungal efficacy of SN in combination with NYT or CHG against biofilms of *C. albicans* or *C. glabrata*. The structure of *Candida* biofilms was also observed using Scanning Electron Microscopy (SEM). The hypothesis of this study is that SN combinations with either NYT or CHG exhibit synergistic antibiofilm activity, and this activity depends on the species and drug concentrations used.

5.3. Materials and methods

5.3.1. Preparation of silver colloidal nanoparticles

Silver colloidal nanoparticles were prepared as previously described.[16] Briefly, 150 ml of an aqueous solution of silver nitrate ($AgNO_3$ - Merck KGaA, Germany) at 5.0 x 10^{-3} mol/l was reduced by 7.5 ml of a solution of sodium citrate ($Na_3C_6H_5O_7$ - Merck KGaA, Germany) at 0.3 mol/l, followed by stabilization with 7.5 ml of a solution with 1.4 mol/l of ammonia (NH_3 - Merck KGaA, Germany) at 90°C. The previous results of electron micrographs showed spherical silver nanoparticles with average size of 5 nm,[13,16] which were used in this study.

5.3.2. Antifungal drugs

Two traditional antifungal drugs were used in this study: NYT as 100 000 IU/ml (corresponding to 22 720 µg/ml) in Mycostatin (Bristol- Myers Squibb, Uk) and CHG (Perio-Aid, Dentaid SL, Spain) as a 0.12% aqueous solution (corresponding to 1 200 µg/ml). Silver colloidal nanoparticles and the antifungal drugs were diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, USA) to final concentrations of 13.5 and 27 µg/ml for SN, 13.5 and 216 µg/ml for NYT, and 9 and 37.5 µg/ml for CHG. NYT or CHG were combined with silver colloidal nanoparticles at the final concentrations mentioned above, generating a total of eight different combinations: 13.5 SN/13.5 NYT, 13.5 SN/216 NYT, 27 SN/13.5 NYT, 27 SN/216 NYT, 13.5 SN/9 CHG, 13.5 SN/37.5 CHG, 27 SN/9 CHG and 27 SN/37.5 CHG.

5.3.3. Candida strains

Two oral clinical isolates of *Candida* species were tested. *C. albicans* 324LA/94 was provided by the culture collection of Cardiff Dental School, Cardiff, UK, while *C. glabrata* D1 was obtained from the biofilm group of the Centre of Biological Engineering, University of Minho, Braga, Portugal. These strains were stored in vials with plastic beads at -80ºC until needed.

5.3.4. Biofilm formation

All *Candida* strains were grown for 20-24 h at 37ºC in a shaker rotating at 120 rpm in 30 ml of Sabouraud dextrose broth (Liofilchem, Italy) using strains grown on Sabouraud dextrose agar (SDA; Liofilchem, Italy) plates not older than 2 days. After being harvested by centrifugation (6500 g; 5 min), cells were washed twice in phosphate buffered saline (PBS; pH 7, 0.1 M) and resuspended in artificial saliva (prepared according to Lamfon et al.[17]) at a concentration of 10^7 cells/ml, determined by a Neubauer counting chamber. Aliquots of 200 µl from this *Candida* cell suspension were inoculated into each well of 96-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) and incubated for 48 h, at 37ºC, under 120 rpm and the medium was renewed after 24 h. At the end of biofim formation assays (48 h), the medium was aspirated and non-adherent cells were removed by washing with 200 µl of PBS.

5.3.5. Biofilm treatment and quantification

Aliquots of 200 µl of each drug suspension alone and in combination with SN were added to the pre-formed biofilms and the microtiter plates were incubated at

37º C for 24 h (at 120 rpm). Adequate controls (without drugs) were also included.

After 24h of treatment, the resulting biofilms were subsequently washed once with 200 µl of PBS and evaluated by total biomass quantification using the crystal violet (CV) staining methodology, as previously described.[13] Briefly, 200 µl of 99% methanol (Romil, UK) were added to the biofilms for 15 min to allow cell fixation. After removing the methanol and drying the microtiter plates at room temperature, 200 µl of CV stain (1% v/v) (Merck, Germany) were added into the wells. The excess of CV was removed after 5 min of contact and, then, 200 µl of acetic acid (33% v/v) (Pronalab, Portugal) were pippeted into the wells in order to dissolve and remove the CV stain from the biofilms. Finally, absorbance was measured in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570 nm.

Additionally, the number of colony forming units (CFUs) of the resulting biofilms was also determined ($\log_{10}$ CFU/cm^2). For that, *Candida* biofilms were scraped from the wells and 10 µl of serial dilutions (in PBS) of the resulting cell suspension were plated (in triplicate) on SDA. The plates were incubated for 24 h at 37ºC to enable CFU enumeration. All assays were carried out at least three times on different days.

5.3.6. Interpretation of drug combinations

The effect of drug combinations was interpreted according to Miceli et al.[9] The combinations were defined as synergistic if a specific combination of SN and an antifungal drug showed a significant reduction ($P < 0.05$) in the biofilm biomass

and cultivable counts of *Candida* biofilm cells compared to the two drugs alone. However, when the combination showed a reduction on *Candida* biofilms compared to the drugs alone but this reduction was not significant, the effect was classified as additive. Finally, an antagonistic effect was identified when the drugs alone produced a reduction significantly higher in the biofilm biomass and cultivable counts of cells than in combination with SN.

5.3.7. SEM analysis

SEM was used to observe the structure of *Candida* biofilms formed within the 24-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) untreated and treated with SN or in combination with NYT or CHG, as described above. For this analysis, biofilms were fixed and dehydrated in a series of ethanol solutions (70% for 10 min, 95% for 10 min and 100% for 20 min), and the microtiter plates were kept in a desiccator. Sections (1 x 1 cm) of the wells bottom were cut with a scalpel blade, mounted onto aluminum stubs, sputter coated with gold and observed under a S-360 scanning electron microscope (Leo, Cambridge, USA).

5.3.8. Statistical analysis

Data were analyzed with SPSS software (SPSS - Statistical Package for the Social Sciences, Inc., Chicago), using ANOVA followed by Bonferroni multiple comparison post-hoc test. P values < 0.05 were considered statistically significant.

5.4. Results

5.4.1. Biofilm quantification

According to the results (Fig. 1A) and the classification based on Miceli et al.,[9] no synergistic effect was observed for all drugs combinations tested regarding to the total biomass reduction of *C. albicans* 324LA/94 biofilms. Their effects were only additive. When compared to the control group, the combinations 13.5 SN/37.5 CHG and 27 SN/37.5 CHG revealed the highest reductions (~70%, $P < 0.001$) in total biomass (Fig. 1A). Generally, the combinations of SN with CHG showed higher reductions in total biomass than the combinations of SN with NYT. However, all combinations of SN with CHG reduced significantly ($P < 0.05$) the total biomass when compared to SN (13.5 and 27 µg/ml) alone, but not in comparison to CHG (9 and 37.5 µg/ml) alone, due to a better effect of CHG in comparison to SN alone (Fig. 1A).

For *C. glabrata* D1 biofilms (Fig. 1B), all combinations of SN with NYT and the combination 13.5 SN/9 CHG accomplished the criteria for synergistic activity. The combination of SN at 27 µg/ml and CHG at 9 µg/ml revealed a significant decrease in the total biomass compared to 27 µg/ml SN alone ($P < 0.001$), but not compared to 9 µg/ml CHG alone ($P = 0.105$), characterizing an additive effect. Furthermore, when compared to the untreated control biofilm, all drugs combinations resulted in a very similar antifungal activity, achieving decreases in the biofilm biomass above 84% (Fig. 1B).

Fig. 2 shows the number of viable cells (in $\log_{10}$ CFU/cm^2) recovered from *Candida* biofilms after treatment with drugs alone and in combinations. SN at

13.5 µg/ml when combined with NYT or CHG revealed a synergistic effect against *C. albicans* 324LA/94 biofilm cells (Fig. 2A). Synergism was also found with the combinations of 27 SN/216 NYT and 27 SN/37.5 CHG, while all other drug combinations showed an additive effect (Fig. 2A). The greatest decrease in $\log_{10}$ CFU/cm^2 (> 6 $\log_{10}$ CFU/cm^2; $P < 0.001$) was achieved by the combination 27 SN/216 NYT when compared to the control group.

For *C. glabrata* D1 biofilms (Fig. 2B), SN at either 13.5 µg/ml or 27 µg/ml in combination with 9 µg/ml CHG showed synergistic activity, while all other drug combinations showed an additive effect. For the drug combinations with additive effect, significant differences ($P < 0.05$) were found only in relation to SN (13.5 and 27 µg/ml) alone, evidencing the better antibiofilm effect of 13.5 µg/ml NYT, 216 µg/ml NYT and 37.5 µg/ml CHG compared to the alone SN effect. When compared to the control group, the higher decreases in the number of biofilm cells were achieved for SN at 13.5 µg/ml in combination with NYT at either 13.5 µg/ml or 216 µg/ml (~5 $\log_{10}$ CFU/cm^2; $P < 0.001$). Surprisingly, the combination of the lowest SN and NYT concentrations tested (13.5 µg/ml for both agents) was the most effective or at least similar to the others (Fig. 2B), contrary to what happened with C. *albicans* 324LA/94 biofilms, where the combination with the highest concentrations showed more antifungal effect (Fig. 2A).

5.4.2. Structure of biofilms

As shown in Fig. 3Ia, untreated *C. albicans* 324LA/94 biofilm displayed a dense network of oval and elongated fungal cells entangled in a thick extracellular matrix. When treated with 13.5 µg/ml SN no significant morphological alteration

was observed (Fig. 3Ie). However, when treated with 27 µg/ml SN (Fig. 3Ib) and 216 µg/ml NYT alone (Fig. 3Ic), the resulting biofilms showed a slightly less compact structure than the control, and it was more evident in the group treated with NYT, suggesting a less amount of extracellular matrix. The treatment with 37.5 µg/ml CHG alone (Fig. 3If) resulted in a biofilm composed of a sparse monolayer of yeasts and hyphae covering the surface. Surprisingly, in the biofilms treated with 27 SN/216 NYT (Fig. 3Id), the fungal cells synthesized larger amounts of extracellular polymeric material and were hidden by the matrix, resulting in a very compact biofilm. Contrary, the major *C. albicans* 324LA/94 biofilm disruption was seen with the combination 13.5 SN/37.5 CHG (Fig. 3Ig). This resultant biofilm displayed few yeasts, deformed and elongated hyphae, as well as some clusters of SN were also visible.

With respect to the ultrastructure of *C. glabrata* D1 biofilms (Fig. 3II), in the control group it was possible to note that the biofilm was composed entirely of a yeast multilayer (Fig. 3IIa). Interestingly, after the treatment with 13.5 µg/ml SN (Fig. 3IIb) and 27 µg/ml SN (Fig. 3IIe), the biofilms showed a more compact structure, composed by a thinner layer of yeasts than the control group. However, after treatment with 13.5 µg/ml NYT alone (Fig. 3IIc), the biofilm presented a less compact layer of yeasts than the groups treated with SN alone. On the other hand, when treated with 9 µg/ml CHG alone (Fig. 3IIf) and 13.5 µg/ml SN in combination with 13.5 µg/ml NYT (Fig. 3IId), the resulting biofilms were formed by aggregated cells and monolayer of yeasts, respectively. Moreover, these biofilms presented a discontinuous distribution covering partially the surface. Finally, the combination 27 SN/9 CHG resulted in a biofilm completely disrupted

and formed by few sparse yeasts (Fig. 3IIg).

5.5. Discussion

Patients with *Candida*-associated denture stomatitis and other oral fungal infections are commonly treated with polyene antifungals, such as NYT.[18,19] Also, CHG is an appropriate adjunct that is effective in the control of oral candidosis.[19] However, these oral conditions are difficult to treat due to the frequent recurrence of the disease[20] and to the biofilm resistance to conventional antifungal therapy.[2] This fact supports the need of investigations on alternative agents or combinations of drugs, like the use of SN with antifungal drugs. The use of combinations with lower drug concentrations can increase the drug efficacy and reduce the adverse effects of these drugs.[21] Thus, this study was designed to evaluate the synergism of SN with NYT or CHG against *C. albicans* and *C. glabrata* biofilms.

In the current study the hypothesis was accepted since SN combined with both NYT and CHG demonstrated synergistic antibiofilm activity, and this activity was dependent on the species and the drug concentrations tested. The silver concentrations selected for this study were those immediately below the minimum concentration that displayed a significant effect on *Candida* biofilms, when SN were tested alone.[13] For NYT, the concentrations used are representative of the maximum (216 μg/ml) and intermediate (13.5 μg/ml) concentrations tested in a pilot study, which showed no differences between these concentrations in the effect against *Candida* biofilms. Finally, for CHG, the concentrations tested were based on another pilot study which was found that the concentration of 37.5 μg/ml was the minimum inhibitory concentration for the sessile cell of the biofilms. Therefore, the highlight of using combinations of those compounds (SN and NYT

or CHG) would be the possibility to obtain better antifungal efficacy in much lower concentrations.

The drug combinations were more effective in reducing the total biomass of *C. glabrata* D1 than *C. albicans* 324LA/94 biofilms (Fig. 1). For *C. glabrata* D1, all combinations of SN with NYT, and the combination of 13.5 SN/9 CHG were synergistic, achieving biomass reductions around 84%. It was noted in a recent study that SN alone were also effective in decreasing biofilm biomass of *C. glabrata* strains.[13] As it has been reported in previous studies,[22,23] *C. glabrata* biofilms present reduced thickness, are devoid of hypha and are less complex and profuse than *C. albicans* biofilms. These features may have facilitated the action of drug combinations in decreasing total biomass of *C. glabrata* biofilms.

As it is noted in Fig. 1, CHG alone showed better results than SN and NYT alone. However, regarding to the number of biofilm cultivable cells (Fig. 2), NYT alone produced the best effects when compared to SN and CHG alone. Interestingly, the combination of SN and NYT presented a synergistic effect in reducing the total biomass (Fig. 1B), while the combination of SN and CHG met the criteria for synergism against the number of *C. glabrata* D1 biofilm cells (Fig 2B). It was not surprising the lack of correlation between both methods used for biofilm quantification, since the two evaluation methods carried out in this study assessed different biofilm features. While the first assay was an indirect quantification of total biofilm biomass (extracellular matrix, and both living and dead cells),[24] the second one determined the number of cultivable cells. CV staining and CFU enumeration were elected because they are generally accepted as complementary methods for biofilm quantification, namely in microtiter plate

assays. In fact, the use of two quantification methods increases the significance of information about antibiofilm agents.

On the other hand, it was surprising to find that keeping constant the concentration of both traditional antifungal drugs (NYT and CHG), the increase of the silver concentration did not reduce significantly the number of CFU (Fig. 2). This fact highlights the possibility of using lower concentrations of SN when combined with NYT and CHG, which is of major importance in reducing silver toxicity. The total biomass and biofilm cultivable cells evaluation was complemented by SEM observations (Fig. 3) which showed that *Candida* biofilms were vastly disrupted (compared to the drugs alone or controls) when treated with the combinations 13.5 SN/37.5 CHG (Fig. 3Ig), 13.5 SN/13.5 NYT (Fig. 3IId) and 27 SN/9 CHG (Fig. 3IIg). Unexpectedly, although the combination 27 SN/216 NYT had produced the highest reduction in the number of CFU for *C. albicans* 324LA/94 biofilms (Fig. 2A), Figure 3Id shows a compact biofilm with high amount of extracellular polymeric material. This corroborates the results found for the total biomass (Fig. 1A), and may indicate that although cells have remained, probably protected by the matrix, their viabilities were reduced. Moreover, although the combinations 27 SN/216 NYT (Fig. 2a) and 13.5 SN/13.5 NYT (Fig. 2b) had been more effective to kill biofilm cells than the combinations 13.5 SN/37.5 CHG (Fig. 2a) and 27 SN/9 CHG (Fig. 2b), respectively, the SEM images suggest that a higher amount of dead cells stayed attached to the microtiter plate surface when *Candida* biofilms were treated with 27 SN/216 NYT (Fig. 3Id) and 13.5 SN/13.5 NYT (Fig. 3IId) than with 13.5 SN/37.5 CHG (Fig. 3Ig) and 27 SN/9 CHG (Fig. 3IIg). So, although SEM images are not representative of the

total biofilm sample, we may hypothesize that the combinations of SN with CHG have a higher capacity to remove biofilm cells formed on microtiter plates than the combinations of SN with NYT.

The synergism found between silver colloidal nanoparticles and NYT or CHG could be explained according to their mechanisms of action. Both NYT and CHG act by disruption of the yeast cell membrane, resulting in alteration of cell permeability and leakage of cell constituents.[5,25] On the other hand, the mechanism of action of SN is multifactorial. Nanoparticles can bind to sulfur-containing proteins resulting in defects in the cell membrane, interact with phosphorus containing compounds like DNA (preventing cell reproduction), and attack the respiratory chain causing the cell death.[10,11] Additionally, the high surface to volume ratio of SN provides better interaction between particles and cells.[26] Thereby, it is possible that the synergism found is due to both different and similar targets of the drugs on the biofilm cells. In this context, the disruption of the cell membrane by NYT and CHG may have facilitated the entry of SN into the cytoplasm, where these particles bind to their targets.[27]

From a clinical perspective, the results of this study are particularly relevant because the NYT and CHG concentrations used in combination with SN are much lower (32-1682 folds) than those found in commercial products Mycostatin and Perio-Aid. Moreover, there was no antagonism for combinations tested and the effect of drug combinations was at least additive. Finally, some of these drug combinations may disrupt *Candida* biofilms, contribute to the prevention of resistance and improve the treatment of *Candida*-associated denture stomatitis.

5.5.1. Limitations

Only one strain of each *Candida* species was evaluated. Furthermore, this study did not examine the effects of drug combinations over a broader range of concentrations, allowing the establishment of fractional inhibitory concentration index. Finally, to confirm the possible role of this combination therapy for treating denture stomatitis, the evaluation of cyto/genotoxicity of these drug combinations is also required.

5.6. Conclusion

In conclusion, this is the first report evidencing the potential of combining SN with either NYT or CHG for improve the antifungal activity against two clinically relevant *Candida* species in biofilm mode of growth.

Acknowledgements

We thank David Williams (Cardiff University, Cardiff, UK) for providing the strain 324LA/94. The authors also thank CAPES (grant BEX 1221/10-8) and FAPESP (2009/15146-5), Brazil, for supporting Douglas Roberto Monteiro work. Silver colloidal nanoparticles used herein were prepared and characterized by LIEC-CMDMC and INCTMN/FAPESP-CNPq, São Carlos, Brazil.

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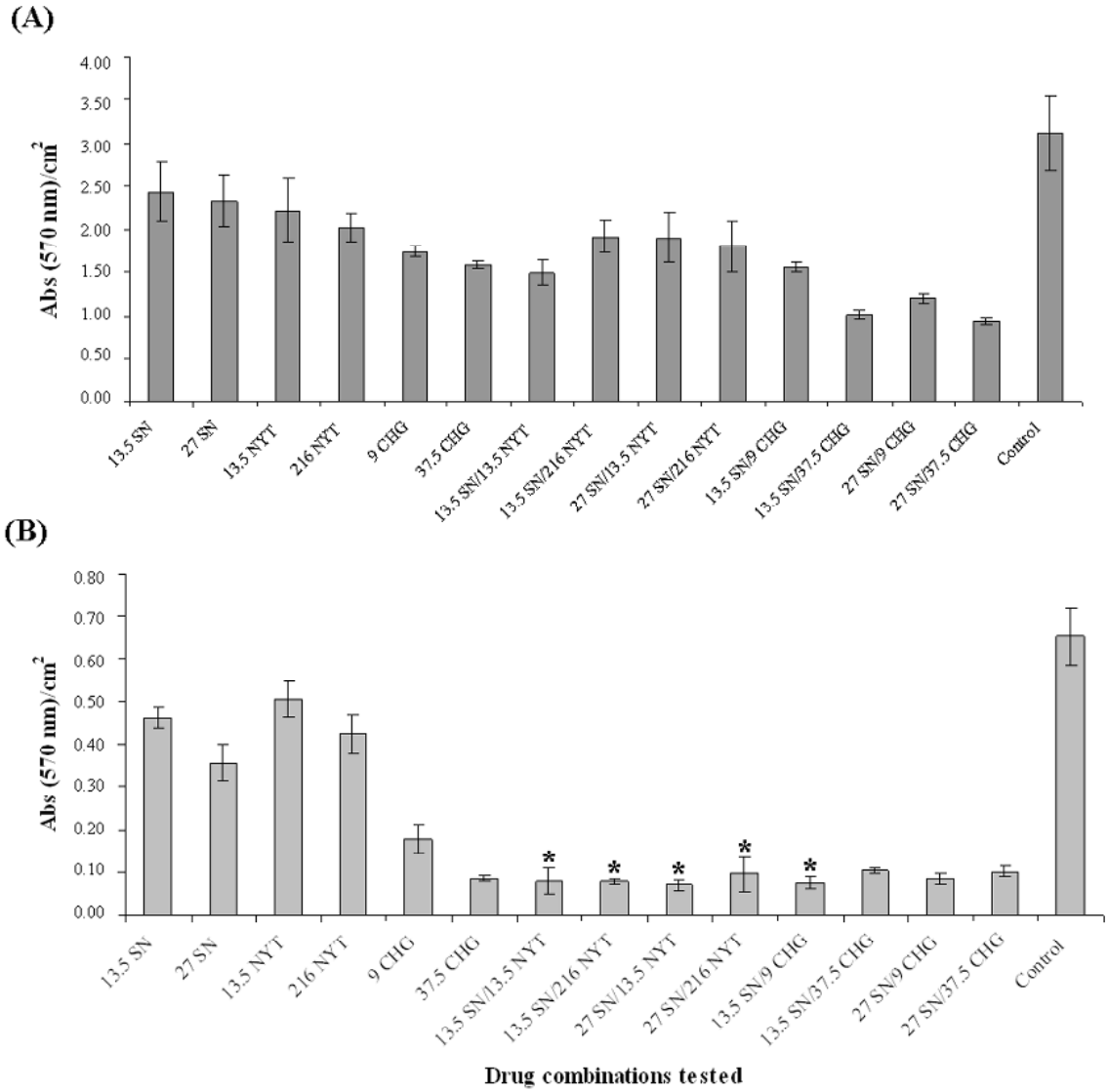

Figure 1. Average absorbances per cm^2 obtained with crystal violet assay for pre-formed biofilms of *C. albicans* 324LA/94 (A) and *C. glabrata* D1 (B) exposed to silver nanoparticles (SN), nystatin (NYT) and chlorhexidine digluconate (CHG) alone and in combination during 24 h. Error bars show standard deviation of the means. *Significant difference ($P < 0.05$, Bonferroni test) in the effect of drug combination (synergistic) compared with the respective drugs alone

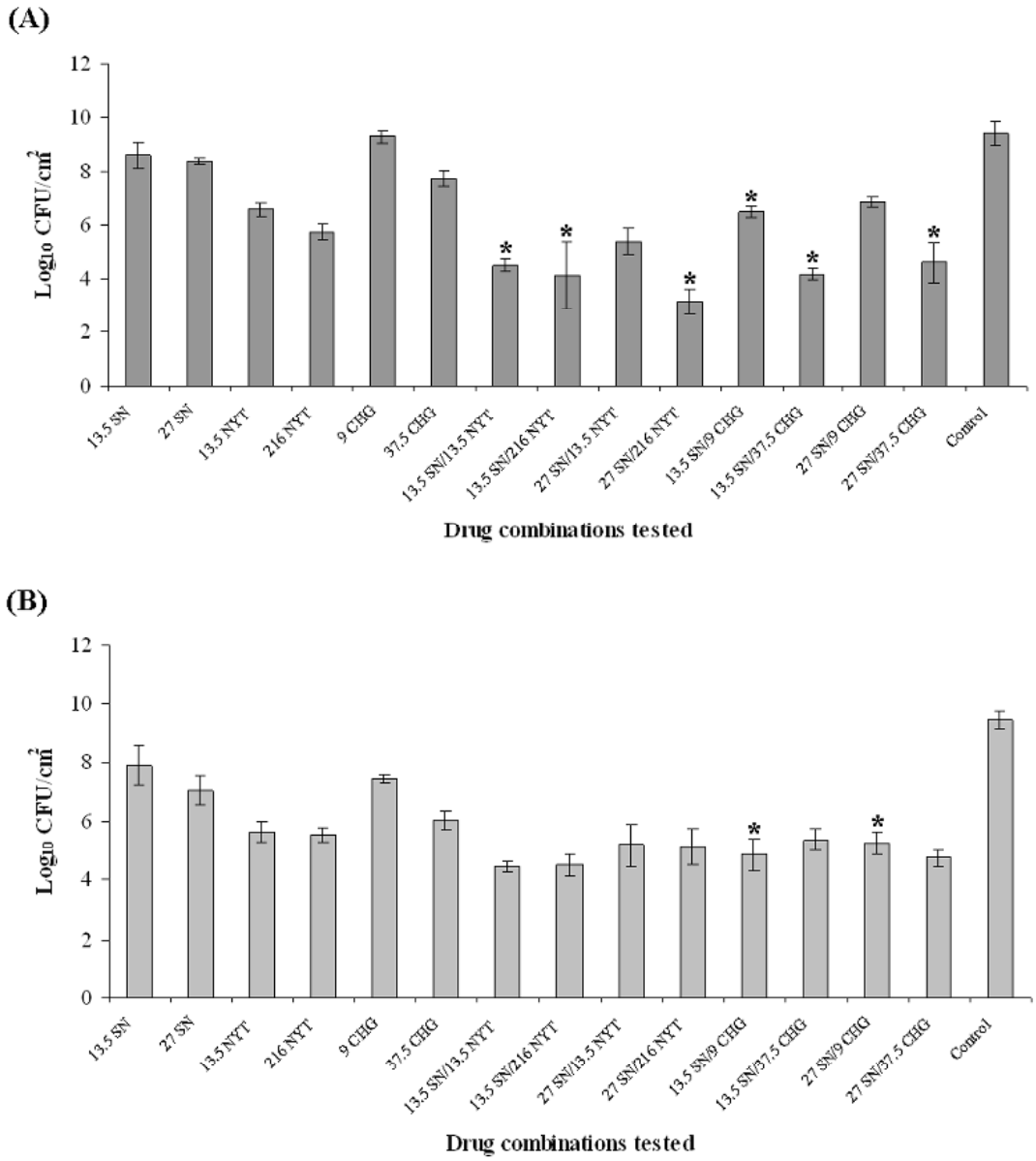

Figure 2. Logarithm of the number of colony forming units per cm^2 of pre-formed biofilms of *C. albicans* 324LA/94 (A) and *C. glabrata* D1 (B) exposed to silver nanoparticles (SN), nystatin (NYT) and chlorhexidine digluconate (CHG) alone and in combination during 24 h. Error bars show standard deviation of the means. *Significant difference ($P < 0.05$, Bonferroni test) in the effect of drug combination (synergistic) compared with the respective drugs alone

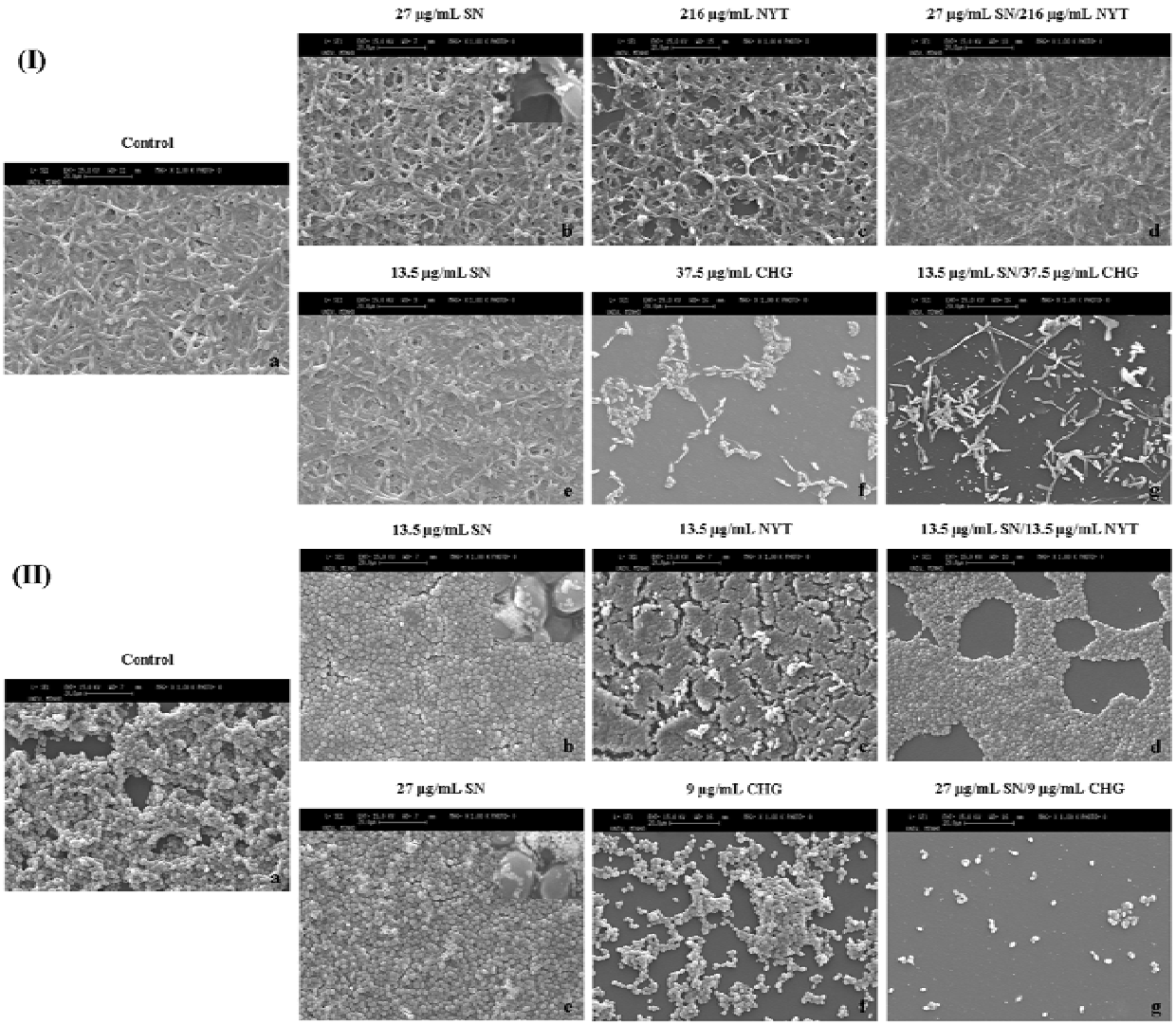

Figure 3. (I) Scanning electron microscopy (SEM) observation of the structure of *C. albicans* 324LA/94 biofilms after treatment with silver nanoparticles (SN), nystatin (NYT) and chlorhexidine digluconate (CHG). (a) untreated biofilm (control), (b-g) biofilms treated with 27 µg/ml SN, 216 µg/ml NYT, 27 µg/ml SN/216 µg/ml NYT, 13.5 µg/ml SN, 37.5 µg/ml CHG and 13.5 µg/ml SN/37.5 µg/ml CHG. (II) SEM observation of the structure of *C. glabrata* D1 biofilms after treatment with SN, NYT and CHG. (a) untreated biofilm (control), (b-g) biofilms treated with 13.5 µg/ml SN, 13.5 µg/ml NYT, 13.5µg/ml SN/13.5 µg/ml NYT, 27 µg/ml SN, 9 µg/ml CHG and 27 µg/ml SN/9 µg/ml CHG. Note clusters of SN in an enlarged view of part of biofilms (images Ib, Ig, IIb and IIe)

CAPÍTULO 5

Adhesion of *Candida* cells to human epithelial cells and polystyrene after treatment with silver nanoparticles[*]

[*]*Artigo enviado para publicação no periódico Journal of Basic Microbiology*

Adhesion of *Candida* cells to human epithelial cells and polystyrene after treatment with silver nanoparticles

6.1. Abstract

The purpose of this study was to determine whether viable cells recovered from *Candida* biofilms treated with silver nanoparticles exhibit reduced adhesion capacity to human epithelial cells and polystyrene surface. *Candida* biofilms were formed in 6-well polystyrene microtiter plates and treated with silver nanoparticles at concentrations of 13.5 and 54 mg l^{-1}. After, biofilms were scraped from the wells and resuspended in phosphate buffered saline. The cellular suspensions obtained were subjected to the exclusion test of cell viability, using Trypan Blue. These *Candida* cell suspensions were added to HeLa cells monolayers or to empty wells of a 24-well microtiter plate (to study adhesion to polysterene). After 2 h of contact, the adhesion capacity of the yeasts was determined using crystal violet staining. The adhesion capacity of *Candida* viable biofilm cells (pretreated with SN) to epithelial cells and polystyrene surfaces was significantly reduced, compared to the control groups, and this reduction was higher when biofilm cells were pretreated with silver nanoparticles in a concentration of 54 mg l^{-1}. These new findings allow to conclude that silver nanoparticles may induce changes in viable yeasts, which can decrease the dissemination of *Candida* infections.

Keywords: *Candida*; silver nanoparticles; yeast adhesion; epithelial cells; polystyrene

6.2. Introduction

Candida albicans followed by *Candida glabrata* are the major commensal pathogens that cause fungal infections in compromised patients, such as oral and vaginal candidiasis [1]. The pathogenicity of these microorganisms is related to several virulence factors, including their ability to adhere to host epithelial cells and/or inanimate substrates, which is an important precondition for colonization, infection and biofilm formation [2, 3]. Additionally, *Candida* biofilms are resistant to a diversity of antimicrobial agents [3]. Moreover, the fungal cells released from biofilms and which return to the planktonic state may act as a continuous source of disseminated infections.

In the light of these considerations, there is a significant interest in the use of alternative antifungal agents which might decrease or inhibit fungal adhesion capacity, preventing the colonization and infection by *Candida* species. Recently, our research group verified that silver nanoparticles (SN) were more effective in inhibiting biofilm formation when applied in prophylaxis than on pre-formed *Candida* biofilms [4], and that the particle size and the type of stabilizing agent did not interfere in the antifungal activity of SN against those biofilms [5]. Thus, with the purpose of complementing these previous data, in this study we tested the hypothesis that *C. albicans* and *C. glabrata* viable cells recovered from *Candida* biofilms treated with SN exhibit significantly reduced adhesion capacity to human epithelial cells and polystyrene surface.

6.3. Materials and methods

6.3.1. Synthesis of silver colloidal nanoparticles

In this research, the average size of SN used was approximately 5 nm. These nanoparticles were synthesized, stabilized and characterized as described previously [4].

6.3.2. Human epithelial cells

A monolayer of epithelial cells from HeLa cell line with origin in human cervical carcinoma (obtained from Gulbenkian Institute of Science, Lisbon, Portugal) was grown (at 37ºC in 5% CO_2) in Dulbecco's modified Eagle's medium (D-MEM; Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (P/S; Gibco, USA). When the cultures reached 80% of confluence, the cells were detached using a 25% trypsin-EDTA (Gibco, USA) solution. The cell concentration was adjusted to 10^6 cells ml^{-1} with fresh D-MEM without P/S and then transferred to a 24-well microtiter plate. Previously to the adhesion tests, the wells were washed twice with phosphate buffered saline (PBS; pH 7, 0.1 M).

6.3.3. Candida strains and growth conditions

Two *Candida* strains were evaluated. *C. albicans* 324LA/94 and *C. glabrata* D1, which are oral clinical isolates obtained from the culture collection of Cardiff Dental School, Cardiff, UK, and from the Biofilm Group of the Centre of Biological Engineering, University of Minho, Braga, Portugal, respectively. They were grown overnight in Sabouraud dextrose broth (Liofilchem, Italy)

medium at 37ºC and 120 rpm, harvested by centrifugation (8000 rpm for 5 min at 15ºC), and washed twice in PBS. Then, fungal cells were counted using a Neubauer chamber and resuspended in mucin-containing artificial saliva medium [4] to achieve 10^7 cells ml^{-1}.

6.3.4. Biofilm formation and treatment with SN

Candida biofilms were produced in 6-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). Aliquots of 4 ml of standardized cell suspensions, as prepared above, were transferred to each well of a microtiter plate. The plates were incubated for 48 h at 37ºC in an orbital shaker at 120 rpm, and the artificial saliva medium was renewed every 24 h. After the incubation stage, the medium was aspirated and the biofilms were washed with 4 ml of PBS in order to remove loosely adherent cells prior to SN treatment. SN suspensions were diluted in RPMI 1640 medium (Sigma-Aldrich, USA) and the biofilms were then treated with SN at 54 and 13.5 mg l^{-1} for 24 h at 37ºC (at 120 rpm). Appropriate untreated controls were also included.

After treatment with SN, the resulting biofilms were washed as described above and then 1 ml of PBS was added to each well and the biofilms were carefully scraped off the well walls using sterilized cell scrapers (Orange Scientific, Braine-l'Alleud, Belgium). The biofilm suspensions were sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W and gently vortexed for 2 minutes to disrupt the aggregates. Following, for enumeration of viable cells, 0.5 ml of each biofilm suspension were mixed with 0.1 ml of 0.4% trypan blue stain (Sigma-Aldrich, USA). After 5 min, the number of viable cells

was adjusted to a concentration of 10^7 cells ml^{-1} in RPMI 1640 medium, using a Neubauer chamber. These *Candida* cell suspensions were used in the succeeding assays.

6.3.5. Candida biofilm cells adhesion assay

A volume of 0.5 ml of viable yeast suspension prepared as above was added to the wells of the 24-well plate covered with a monolayer of human epithelial cells or to empty wells of 24-well plates (to study adhesion to polystyrene). In both conditions the plates were incubated at 37ºC for 2 h. Next, the wells were washed once with PBS to remove non- adherent yeasts.

The quantification of adhered yeasts to epithelial cells and to the polystyrene surface was carried out by the crystal violet staining method, as described by Negri *et al.* [6] and Monteiro *et al.* [4], respectively. For adhesion assays, the mean absorbance of yeasts was standardized by number of adhered yeasts per area of the well using *C. albicans* 324LA/94 and *C. glabrata* D1 standard curves [6].

6.3.6. Statistical analysis

In all cases, the assays were performed in triplicate on three separate occasions. The results were analyzed using ANOVA with Bonferroni test. A P value $< .05$ was taken to be statistically significant. The statistical analysis was obtained in the SPSS software (SPSS - Statistical Package for the Social Sciences, Inc., Chicago).

6.4. Results and discussion

As it is possible to observe in Fig. 1a and b, compared to the control groups (biofilm cells without SN treatment), *C. albicans* 324LA/94 and *C. glabrata* D1 biofilm cells showed reductions in the capacity to adhere to HeLa cells around 53% (P = .001) and 34% (P < .001) when pretreated with silver at 13.5 mg l^{-1} and around 40% (P = .003) and 86% (P < .001) at a silver concentration of 54 mg l^{-1}, respectively.

The number of yeast cells adhered to polystyrene surface is presented in Fig. 1c and d. A significant lower number of adhered cells was verified after pretreatment of biofilm cells with 13.5 and 54 mg l^{-1} of silver compared to controls. Reductions around 70% (P < .001) and 46% (P = .006) were found when *C. albicans* 324LA/94 and *C. glabrata* D1 biofilm cells were pretreated with 54 mg l^{-1} of silver, respectively. While for *C. albicans* 324LA/94 the adhesion inhibition is dose dependent, for *C. glabrata* D1 the pretreatment with the higher concentration of silver showed no significant difference in the adhesion capacity when compared to the group pretreated with the lower concentration (P > .05). It is worth noting that the silver concentrations used herein were unable to completely eradicate *C. albicans* 324LA/94 and *C. glabrata* D1 biofilms [4, 5].

The adhesion capacity of yeasts is related to several factors, including connection of cell wall adhesins to specific binders [7], electrostatic interactions (zeta potential) between microbial cells and substrate surfaces, and their hydrophobicity [8]. As several factors are implicated in the adhesion process, the understanding of the results obtained in this study is difficult. However, we believe that, probably, SN interfered with the expression or synthesis of adhesins

on the viable yeasts [9, 10]. The reduction in adhesion could also have occurred due to alterations on other properties of live yeast cell surfaces generated by the action of these nanoparticles.

Furthermore, this study demonstrated that the amount of adhered yeasts varies according to the strain and the type of substrate (epithelial cells and polystyrene surface). For *C. albicans* 324LA/94, the groups untreated and pretreated with SN at 13.5 mg l^{-1} showed a significantly higher capacity to adhere to polystyrene than to HeLa cells ($P < .001$). At a SN concentration of 54 mg l^{-1} there was no significant difference in the amount of adhered yeasts to HeLa cells and to polystyrene ($P = .211$). Interestingly, for *C. glabrata* D1, it was possible to verify that the control group and the group pretreated with SN at 13.5 mg l^{-1} demonstrated a significantly higher capacity to adhere to HeLa cells than to polystyrene ($P < .001$). However, when biofilm cells were pretreated with SN at 54 mg l^{-1}, the extent of adhesion to polystyrene was significantly higher ($P = .005$).

Importantly, this work revealed the ability of SN to affect an important virulence factor of *Candida* species - surface colonization. Thus, the findings of the present study taken together with our previous results [4, 5] demonstrate an obvious dual function of SN: anti-adhesion and antifungal effects against *C. albicans* 324LA/94 and *C. glabrata* D1.

In conclusion, our results sustain the hypothesis that the pretreatment of *Candida* biofilm cells with SN significantly reduces the subsequent adhesion capacity of *C. albicans* 324LA/94 and *C. glabrata* D1 viable cells to human epithelial cells and polystyrene surface. These new findings highlight the potential

use of SN in controlling the dissemination of *Candida* infections, especially in susceptible patients, like those undergoing cancer chemotherapy. However, additional studies with a wide number of strains and directed to investigate the accurate mechanisms by which adhesion capacity of *Candida* cells is decreased after treatment with SN are needed.

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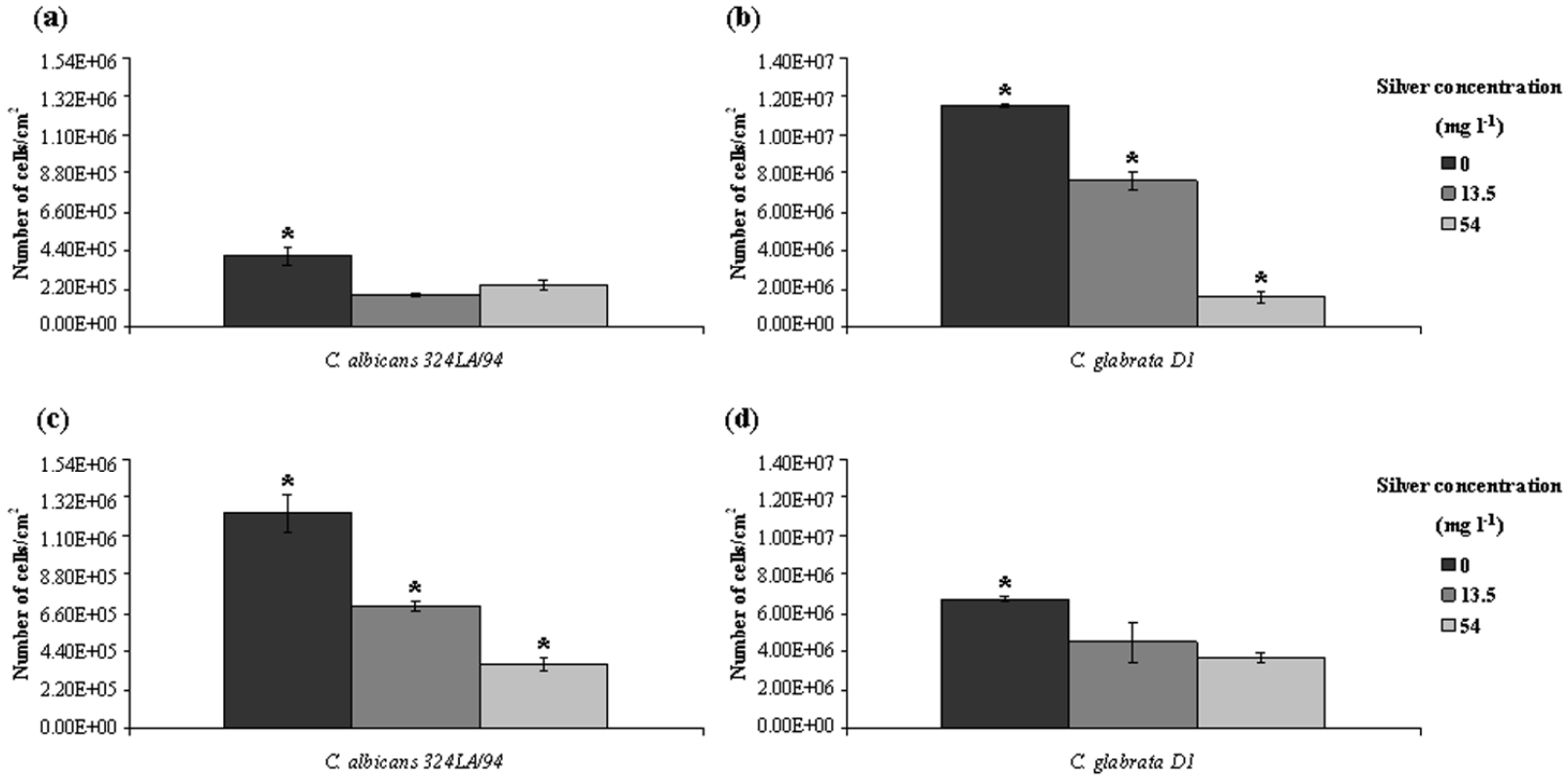

Figure 1. Adhesion capacity (2 h) of viable cells recovered from *Candida* biofilms pretreated with two different silver nanoparticles concentrations (13.5 and 54 mg l^{-1}) to HeLa cells (a and b) and polystyrene surface (c and d), measured by crystal violet staining assay and standardized as cells cm^{-2}. Error bars symbolize the standard deviations of the means. *$P < .05$ between the different pretreatments for the same species

CAPÍTULO 6

Silver colloidal nanoparticles: influence of chemical stability on *Candida* biofilms formed on denture acrylic[*]

[*]*Artigo nas normas do periódico Journal of Antimicrobial Chemotherapy*

Silver colloidal nanoparticles: influence of chemical stability on *Candida* biofilms formed on denture acrylic

7.1. Abstract

Objectives: To assess the effect of silver nanoparticles (SN) subjected to temperature and pH variations on *C. albicans* and *C. glabrata* biofilms. The influence of different periods of biofilm formation and treatment in the susceptibility of *Candida* biofilms to SN were also evaluated. **Methods:** Colloidal suspensions of SN (5 nm) were heated for 30 min at 50, 70 and 100ºC. In a separate experiment, the pH of these suspensions was adjusted to 5.0 and 9.0, and after 2 h the pH was readjusted to 7.0. Then, these suspensions were used to treat for 24 h each *Candida* biofilm grown on acrylic surface. The efficacy of these SN was determined by the quantification of total biomass and colony forming units (CFUs). Further, the antifungal activity of stock solution of SN at a defined concentration of 54 mg/L was also tested against biofilms grown for both 24 and 48 h and treated for two different periods (5 and 24 h). **Results:** The preheating of SN suspension at 100ºC and the pH variation only affected adversely the biomass of *C. albicans* 324LA/94 and *C. glabrata* strains, respectively. The period of biofilm formation revealed a significant influence on the susceptibility to SN on the viability of *C. albicans* 324LA/94. The comparison between 5 and 24 h of treatment was significantly different for the *C. glabrata* ATCC biomass and for the *C. albicans* 324LA/94 viability, with best results for the shortest treatment period. **Conclusions:** Although the SN chemical stability as well as the different periods of biofilm formation and treatment have not shown expressive influences on susceptibilities of *Candida* biofilms to SN, the results provided important insights that may be useful for managing denture stomatitis with formulations based on SN.

Keywords: silver nanoparticles; chemical stability; biofilms; *Candida albicans*; *Candida glabrata*.

7.2. Introduction

Candida species are normally present in the oral cavity as commensals,[1] contributing to the ecological equilibrium of the microbiota. However, factors such as diabetes mellitus, nutritional deficiencies, usage of indwelling devices, broad-spectrum antibiotics and corticosteroids, cancer chemotherapy, and human immunodeficiency virus (HIV) infection have facilitated the breakdown of ecological equilibrium and the emergence of *Candida* species as opportunistic pathogens.[2,3]

Candida-associated denture stomatitis is frequent in elderly people and specially those complete denture wearers and considered one of the most common forms of oral candidiasis.[4,5] Besides the predisposing factors mentioned above, its development also involves biofilm formation.[4,5] Even though *Candida albicans* is strongly associated with the development of denture stomatitis, non-*Candida albicans Candida* species, such as *Candida glabrata*, may also contribute to this pathological condition .[6,7] *Candida* species form biofilms on denture acrylic surfaces which are characterized by networks of yeasts, pseudohyphae and hyphae surrounded by an extracellular matrix[8] and embedded mainly into irregularities of acrylic surfaces.[9] Contrary to *C. albicans*, *C. glabrata* do not form hyphae[1] and, interestingly, Luo and Samaranayake[10] observed that *C. glabrata* does not adhere well to oral keratinocytes, but adheres better to denture surfaces, when compared with *C. albicans*.

Although antifungal suspensions based on nystatin, amphotericin B, miconazole and fluconazole have been widely used to treat denture stomatitis,[11,12] *Candida* infections are frequently recalcitrant to conventional treatment.[5,13-15]

Indeed, established *Candida* biofilms are more resistant to antimicrobial agents than their planktonic counterparts.[16,17] The clinical impact of increased resistance to conventional antifungal therapy has encouraged the search for alternative strategies which could contribute significantly in preventing and breaking down *Candida* biofilm formation. For this purpose, silver nanoparticles (SN) have nowadays been recognized as a new class of antimicrobial agent[18] because of their effective antimicrobial ability. SN have large surface area and high reactivity compared with the microparticles.[19] In dentistry, these nanoparticles have been incorporated into dental adhesives[20,21] and resin composites,[22] for inhibition of dental caries, and into denture base acrylic resin[23] in order to prevent denture stomatitis.

The mechanisms of killing microorganisms of SN are multifactorial[18,19,24-26] and it is believed that these mechanisms are similar to those of silver ions (SI).[24,27] In general, (i) SN bind to sulfur-containing proteins in biological molecules resulting in defects in the microbial cell membrane and loss of intracellular contents, (ii) inhibit respiratory chain enzymes and (iii) bind to phosphorus containing compounds like DNA, preventing cell reproduction.[18,19,24-26] The targets of action of SN are relatively well described in complex studies.[18,24-26,28] In particular, the cytotoxic properties of SN which vary according to their size and concentration have also been studied.[29,30]

Recently, it was demonstrated that SN were more effective against *Candida* adhered cells than against mature biofilms.[31] Furthermore, SN with different diameters (5, 10 and 60 nm) formed through the reduction of silver nitrate with sodium citrate and stabilized with ammonia or polyvinylpyrrolidone

(PVP) showed no significant differences in the effect against *C. albicans* and *C. glabrata* mature biofilms.[17]

One essential aspect that remains to be established is the influence of SN chemical stability on their efficacy against *Candida* biofilms. Thus, the main aim of this study was to evaluate the effect of SN subjected to temperature and pH variations on *C. albicans* and *C. glabrata* biofilms. The influence of different periods of biofilm formation and treatment in the susceptibility of *Candida* biofilms to SN were also investigated. The null hypotheses were that (i) neither temperature variation nor pH variation would affect the efficacy of SN against *Candida* biofilms, and (ii) both the different times of biofilm formation and treatment would not affect the susceptibility of *Candida* biofilms to SN.

7.3. Materials and methods

Synthesis and characterization of silver colloidal nanoparticles

SN were synthesized in accordance with a previously described procedure.[32] Briefly, a solution of silver nitrate ($AgNO_3$ - Merck KGaA, Darmstadt, Germany) at 5.0 x 10^{-3} mol/L was reduced by a solution of sodium citrate ($Na_3C_6H_5O_7$ - Merck KGaA) at 0.3 mol/L. The solution increasingly turned amber yellow and a colloidal suspension was formed. After, the silver colloids were stabilized by adding a solution with 1.4 mol/L of ammonia (NH_3 - Merck KGaA). SN suspensions were characterized by UV/Visible spectroscopy (Spectrophotometer Shimadzu MultSpec-1501; Shimadzu Corporation, Tokyo, Japan) and by Transmission Electron Microscopy (TEM, Electron Microscope FEG-VP Supra 35; Carl Zeiss, Jena, Germany). The typical absorption spectrum of SN centered at 430 nm was obtained in the UV/Vis spectrum and the electron micrograph images displayed spherical SN having an average particle size of 5 nm.[17,31] The SN concentration of 54 mg/L used in this study was based on its efficacy against *Candida* biofilms reported in our previous studies.[17,31]

Chemical stability of SN through temperature and pH variations

The chemical stability assays were carried out at roon temperature based on Ellis *et al.*[33] method, with some modifications. For the temperature stability assay, stock suspensions of SN in glass tubes were placed in a water bath at 50 (SN-50), 70 (SN-70), and 100ºC (SN-100) for 30 minutes. Later, SN suspensions (pre-heated) were diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) 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-pH5) or pH 9.0 (SN-pH9) using solutions of 1 M HCl and 1 M NaOH, 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; Polidental Ind. e Com. Ltd., São Paulo, Brazil) on a glass plate with rough surface (to mimic the roughness palatal) and invested in metallic flask with type III dental stone (Herodent; Vigodent, Petrópolis, Brazil). After the dental stone had set, the flask halves were separated, the wax was removed, and the matrix molds and the glass plate were cleansed. 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 x 10 x 3 mm) were deflasked and the excess of 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 finally autoclaved at 121°C for 15 min.[8]

Artificial saliva medium

The medium used for biofilm formation was artificial saliva (AS, pH 6.8)[34] and its composition per 1 L of deionized water was: 2 g of yeast extract (Sigma-Aldrich,

St Louis, USA), 5 g of peptone (Sigma-Aldrich), 2 g of glucose (Synth; Labsynth Produtos para Laboratórios Ltd., Diadema, Brazil), 1 g of mucin (Sigma-Aldrich), 0.35 g of NaCl (Merck KGaA), 0.2 g of $CaCl_2$ (Sigma-Aldrich) and 0.2 g of KCl (Merck KGaA).

Yeast strains and growth conditions

Two different strains of *C. albicans* and *C. glabrata* were tested. In addition to the reference *C. albicans* (ATCC 10231) and *C. glabrata* (ATCC 90030) strains, two *Candida* oral clinical isolates were used in this work, 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 dextrose agar medium (SDA, Difco; Becton, Dickinson & Co., Le Pont de Claix, France) at 37º C for 24 h. A loopful of the *Candida* colonies from SDA plates was inoculated into Sabouraud dextrose broth (SDB; Difco) medium and incubated at 37ºC for 20-24 h under agitation (120 rpm). After being harvested by centrifugation (8000 rpm for 5 min at 15ºC), cell pellets were washed twice in phosphate buffered saline (PBS; pH 7, 0.1M) and the cellular density adjusted to 1 x 10^7 cells/mL in AS, using a Neubauer haemocytometer. These *Candida* cell suspensions were used in the subsequent biofilm formation assays.

Influence of SN chemical stability on their efficacy against Candida biofilms

Biofilm formation and treatment with SN

Candida biofilms were formed on acrylic resin specimens according to Silva *et al*.[8] Briefly, acrylic specimens were placed in 24-well microtiter plates (Costar, Corning Inc. Life Sciences, Tewksbury, MA, USA) and 1 mL of the standardized *Candida* cell suspension (1 x 10^7 cells/mL in AS) was added into each well and incubated at 37ºC for 48 h under agitation (120 rpm). After 24 h, 500 µL of AS were 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 non-adherent cells. Then, 1 mL of 54 mg/L of SN (with temperature and pH variations, as previously described) diluted in RPMI 1640 medium was added to the pre-formed *Candida* biofilms and incubated at 37ºC for 24 h under agitation (120 rpm). For positive and negative controls, *Candida* biofilms were pre-formed on acrylic specimens and then incubated with 54 mg/L of SN without temperature and pH variations and with RPMI 1640 medium without SN, respectively.

Quantification of total biofilm biomass

Total biomass of *Candida* biofilms exposed to SN (with or without variation of temperature and pH) was measured using the crystal violet (CV) staining method.[8,17,31] 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 into each well containing acrylic specimens and incubated for 5 min. Acrylic specimens were

gently washed with deionized water, dried at room temperature, transferred to new 24-well microtiter plates, and 1 mL of acetic acid (33% v/v) (Sigma-Aldrich) added to remove the CV stain from the biofilms. Lastly, absorbance of the obtained solution was measured in a microtiter plate reader (Eon Microplate Spectrophotometer; Bio Tek, 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 cells

Candida cultivable cells from biofilms exposed to SN (with or without variation of temperature and pH) 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 SDA, and the plates were incubated at 37ºC. After 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 three times in triplicate.

Influence of different periods of biofilm formation and treatment in the susceptibility of Candida biofilms to SN

Biofilm formation and treatment with SN

Candida biofilms were grown on acrylic resin surfaces for 24 and 48 h, according to the procedure described above, except that for 24-h biofilms the AS medium was renewed after 12 h. Literature data[14] reveal three development phases of

Candida biofilms on polymethylmethacrylate strips: early (0-11 h), intermediate (12-30 h), and maturation (31-72 h). Therefore, this study was carried out with 24 h- and 48 h-biofilms to include the intermediate and maturation phases. The effect of SN on adhered cells (early phase) was demonstrated in a previous study.[31] After the times of biofilm formation (24 and 48 h), AS was removed, the acrylic specimens were washed once with 1 mL of PBS, and 1 mL of RPMI 1640 medium containing SN diluted at 54 mg/L (without temperature and pH variations) was transferred to the wells of the 24-well microtiter plates containing 48-h biofilms on the denture acrylic surfaces. Controls devoid of SN were also included. Finally, the microtiter plates were incubated at 37ºC with shaking (120 rpm) for 5 and 24 h. After each period of treatment (5 and 24 h), acrylic specimens were washed once with 1 mL of PBS to remove non-adherent cells.

Biofilm quantification

The influence of different periods of biofilm formation and treatment on the susceptibility of *Candida* biofilms to SN was evaluated by CV staining and CFUs enumeration, as described previously. All assays were also carried out in triplicate and on three separate occasions.

Statistical analysis

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

7.4. Results

Influence of SN chemical stability on their efficacy against Candida biofilms

Quantification of total biofilm biomass

Figures 1A and 2A show the effect of temperature and pH of SN colloidal suspension on their efficacy in reducing *Candida* biofilm biomass. It was possible to observe that for *C. albicans* ATCC 10231, *C. albicans* 324LA/94, *C. glabrata* ATCC 90030 and *C. glabrata* D1, the treatment for 24 h with SN with no prior heating and pH variation (positive controls) yielded decreases in total biofilm biomass of 18.5 (P = 0.028), 32.3 (P = 0.021), 22.3 (P = 0.001) and 17.6% (P = 0.027), respectively, when compared to negative controls (treatment without SN).

According to Figure 1A, for *C. albicans* ATCC 10231 and *C. glabrata* D1, the comparisons among the groups SN-50, SN-70 and SN-100 and between each ones with positive and negative controls showed no statistical differences in the biomass reduction (P > 0.05). However, for *C. albicans* 324LA/94 and *C. glabrata* ATCC 90030, increasing the temperature of SN suspension it was possible to note a gradual increase in biofilm biomass with a significant difference between SN-50 and SN-100 (P = 0.04 and P = 0.025, respectively). Even for those *Candida* strains, SN-50 showed significant reduction in the total biomass compared to the negative controls (P = 0.033 and P = 0.006, respectively), on the other hand no significant differences were found when compared to the positive controls (P > 0.05). Only for *C. albicans* 324LA/94 SN-100 produced a significant increase in the total biomass compared to the positive control group (42.9%, P = 0.037).

The results displayed in Figure 2A show that for both strains of *C.*

glabrata the pH variation significantly impaired the efficacy of SN in reducing the total biomass. When the SN-pH5 group was compared to the positive controls, it was observed 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. In addition, changing the pH to 9.0 the total biomass increased about 38% (P = 0.036) for *C. glabrata* ATCC 90030 and 39% (P = 0.009) for *C. glabrata* D1 compared with the positive controls.

Quantification of biofilm cells

Regarding the effect of SN chemical stability on the biofilm cultivable cells, Figures 1B and 2B clearly show that the temperature and the pH variations neither impaired nor improved significantly the efficacy of SN against *Candida* biofilms. Furthermore, only the positive control for *C. glabrata* D1 showed a significant reduction in the number of CFUs (0.71-$\log_{10}$, P = 0.034) in relation to the negative control group.

Influence of different periods of biofilm formation and treatment in the susceptibility of Candida biofilms to SN

Quantification of total biofilm biomass

In accordance with Figure 3A, it is 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 mg/L showed significant reductions in the total biomass of 23 (P = 0.002), 22.9 (P = 0.008), 42.9 (P < 0.001) and 27.7% (P < 0.001), respectively, compared to their respective controls.

However, when these biofilms were treated for 24 h, the reductions in biomass compared to the controls were lower than those produced by the treatment for 5h and were only significant for *C. glabrata* ATCC 90030 (24.1%, $P < 0.001$) and *C. glabrata* D1 (13.9%, $P = 0.027$).

For 48-h biofilms (Figure 3B), *C. albicans* ATCC 10231, *C. albicans* 324LA/94, *C. glabrata* ATCC 90030 and *C. glabrata* D1 treated with SN for 5 h 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. When these biofilms were treated for 24 h, the reductions in the total biomass compared to the control groups were also significant for all strains: *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%, $P = 0.027$).

For 24- and 48-h biofilms (Figure 3), although the treatment for 5 h has resulted in a higher reduction in total biomass than the treatment for 24 h, the difference between these treatments for the same age of biofilm growth was only significant for *C. glabrata* ATCC 90030 ($P < 0.001$, 24-h biofilms; $P = 0.001$, 48-h biofilms). For the same treatment (5 or 24 h), the period of biofilm formation did not interfere on the susceptibility of *Candida* biofilms to SN ($P > 0.05$).

Quantification of biofilm cells

Concerning the effect of SN on the biofilm cultivable cells (Figure 4A), 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 respectively decreases

in the number of CFUs of 0.27 ($P > 0.05$), 0.96 ($P = 0.014$), 1.59 ($P = 0.007$) and 1.03-$\log_{10}$ ($P = 0.035$), compared to the control groups. When these biofilms were treated for 24 h and compared to the controls, there were reductions in the number of CFUs of 1.18-$\log_{10}$ for *C. albicans* ATCC 10231 ($P = 0.027$), 1.77-$\log_{10}$ for *C. albicans* 324LA/94 ($P < 0.001$), 1.15-$\log_{10}$ for *C. glabrata* ATCC 90030 ($P > 0.05$) and 1.0-$\log_{10}$ for *C. glabrata* D1 ($P = 0.043$). Interestingly, the treatment for 5 h was more effective in reducing the number of CFUs for the *C. glabrata* strains whereas the 24 h-treatment was more effective against *C. albicans* strains. Despite of this, there were no significant differences ($P > 0.05$) between the two treatment periods for all strains tested.

For 48-h biofilms (Figure 4B), the treatments with SN for 5 and 24 h produced significant decreases in the number of CFUs 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 to 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 shortest treatment period (5 h). Additionally, for the same treatment period, the biofilm formation period revealed a significant influence on the susceptibility to SN only for *C. albicans 324LA/94* biofilms treated for 24 h, with higher decrease in the number of CFUs for 24-h biofilm than for 48- biofilm ($P < 0.001$).

7.5. Discussion

The results of the present study did not allow the total acceptance of the null hypothesis that neither temperature changes nor pH variation would affect the SN efficacy against *Candida* biofilms, since the preheating of SN suspension at 100ºC affected adversely the *C. albicans* 324LA/94 biomass, and the pH variation to 5.0 and 9.0 also affected adversely the efficacy of SN in decreasing the total biomass of both stains of *C. glabrata*.

As it is shown in Figure 1, the preheating of SN at 50, 70 and 100ºC had no significant influence on total biomass (Figure 1A) and on cell viability (Figure 1B) of *Candida* biofilms. The only exception to these findings occurred for the biomass of *C. albicans* 324LA/94, where SN heated at 100ºC induced an increase of 42.9% in the total biomass in relation to the no-heating group (positive control). In an attempt to explain these findings, additional tests were performed to verify the behavior of SN suspension when subjected to heating.

Aliquots of each SN suspension heated at 50, 70 and 100ºC were evaluated by UV/Vis spectroscopy. Surprisingly, SN-50 and SN-70 suspensions did not show expressive changes regarding to their spectral absorption aspects when compared with no-heating-SN suspension,[17,31] indicating that these suspensions were still stable after 30 min of heating. This is a good agreement with the similar results of total biomass (Figure 1A) and cultivable biofilm cells (Figure 1B) obtained for SN-5, SN-70 and SN without heating and explains why there were no significant differences among these groups.

Kittler *et al.*[35] evaluated the dissolution in water of SN stabilized with citrate and PVP for up to 125 days at 5, 25 and 37ºC and found that SN dissolved

partially into ions and that an increase in temperature caused an increase in the degree of dissolution and release of SI. As a result of this process, a considerably increased toxicity of SN was also observed. In our study, although the nanoparticles have been heated at high temperatures, the short heating period (30 minutes) may have been insufficient to provide a significant dissolution of these particles with release of large amounts of SI and consequently improving the antibiofilm activity. It must also be emphasized that the SN used herein were well stabilized with ammonia. The role of ammonia is trapping all SI present in the colloidal suspension by forming soluble diamine silver (I) complexes, which prevents the growth of already formed SN and the formation of new nanoparticles.[32]

However, heating the SN suspension at 100ºC changed the suspension color and the particle size distribution, as it reveals Figure 5 by the shift of the absorption peak maximum towards higher wavelength and the peak broadening of the UV-Vis spectra. It might indicate that the SN-100 suspension was significantly destabilized, which led to the formation of silver aggregates or new nanoparticles.[36] However, the efficacy of SN-100 suspension against *Candida* biofilms was significantly reduced only for the *C. albicans* 324LA/94 biomass. Unfortunately, the reason for that is unknown but, what our results point is that fresh stock solution of SN or nanoparticles destabilized by heating may not be crucial when they will be used against *Candida* biofilms. Perhaps, this is due to the inherent tendency of SN agglomeration when in contact with biofilms.[37]

Regarding the tests carried out to evaluate the efficacy of SN suspensions with pH changed, it is important to explain that the pH at 5.0 was chosen because

it is close to the average value of the saliva pH from patients with denture stomatitis,[38] while the pH 9.0 was arbitrarily chosen as representative of basic pH. Interestingly, after adjusting the pH (to 5.0 or 9.0) of SN colloidal suspensions diluted in RPMI 1640 medium, it was observed a few dark aggregates or flocs sedimented on the vessel bottom, indicating instability of the colloidal suspensions at both acidic and basic pH. According to Kittler *et al.*,[35] 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 HCl or NaOH probably led to a destabilization in the particles system and favored the SI release, which may have bound to proteins or precipitated in the RPMI medium as silver chloride,[39] silver phosphate[35] or silver hydroxide.

The results showed in Figure 2B demonstrate that the pH variation did not impair or improve significantly the efficacy of SN on the number of CFUs. Indeed, the reasons for these findings would be related to those mentioned above as regards the effect of the temperature stability on the antifungal potential of SN. However, the pH changes provided significant increases in the total biomass of *C. glabrata* ATCC 90030 and *C. glabrata* D1 when compared to the positive controls (Figure 2A). At this point, as *C. albicans* biofilms are more profuse and produce greater amounts of extracellular matrix than those of *C. glabrata*,[1,40] the nanoparticles and some aggregates were likely retained in the matrix, and consequently did not reach the cells which were in the deeper layers of the biofilm. Thinking in this way, we speculate that in *C. glabrata* ATCC 90030 and

C. glabrata D1 biofilms the contact of aggregates with their cells may have been facilitated. Although it has not resulted in a decrease in the number of viable cells (probably due to SN aggregation), it may have generated a stress response in the biofilm cells, which responded by producing more matrix.

Also the second null hypothesis of this study was not fully accepted, since the different times for biofilm formation only revealed a significant influence on the susceptibility of viable cells of *C. albicans 324LA/94* treated with SN for 24 h. Additionally, comparing the treatment periods (5 and 24 h), there were significance for the biomass of *C. glabrata* ATCC 90030 biofilm (grown for 24 and 48 h) and for the number of CFUs of C. *albicans* 324LA/94 biofilm (grown for 48 h), with better results for the shortest treatment period for all.

Several studies[14,41,42] showed age-related differences of biofilms in response to antimicrobial agents. Chandra *et al.*[14] found that the minimum inhibitory concentrations of amphotericin B, nystatin, fluconazole, and chlorhexidine were significantly higher for mature *C. albicans* biofilms than for early and intermediate biofilms. Another study revealed that mature bacteria biofilms were more resistant than young biofilms to chlorhexidine preparations.[42] In contrast, our results showed that the susceptibilities of biofilms in the two formation phases were almost similar, except for *C. albicans* 324LA/94 (Figure 4A and 4B). For this strain, the treatment with SN during 24 h provided a significantly higher reduction in the number of CFUs for 24-h biofilms than for 48-h biofilms (~2-$\log_{10}$ of difference). These findings may be due to the decreased metabolic activity of fungal cells in mature biofilms[14] and to the diffusion barrier to SN penetration promoted by extracellular matrix. *Candida* biofilms in

intermediate phase are characterized by the emergence of extracellular matrix covering the fungal microcolonies, while mature biofilms exhibit higher amount of extracellular material and fungal cells completely covered within this material.[14] Likely, this result was only observed for *C. albicans* 324LA/94 because the difference between the two biofilm formation phases was more relevant for this strain.

Finally, in general, it was observed that the treatment of *Candida* biofilms with SN during 5 h was more effective than for 24 h (Figures 3 and 4). There is not an exact explanation for this finding. What must be considered is the inherent physiological differences among the species and strains tested[40]. 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 compact monolayer or multilayer of yeasts.[40] These features may hinder or facilitate the action of SN against *C. albicans* and *C. glabrata* biofilms.

In conclusion, our results indicated that the temperature and pH variations of SN affected differently the biomass and the sessile cells of *Candida* biofilms, and for these cells they acted similarly to the SN stock suspension even losing their chemical stability at 100ºC and under acid and basic pHs. Furthermore, in general, the biofilm formation and treatment periods did not seem to be decisive on the susceptibility to SN. These results may help guide therapeutic decisions with formulations containing SN in patients with *Candida*-associated denture stomatitis.

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7.6. References

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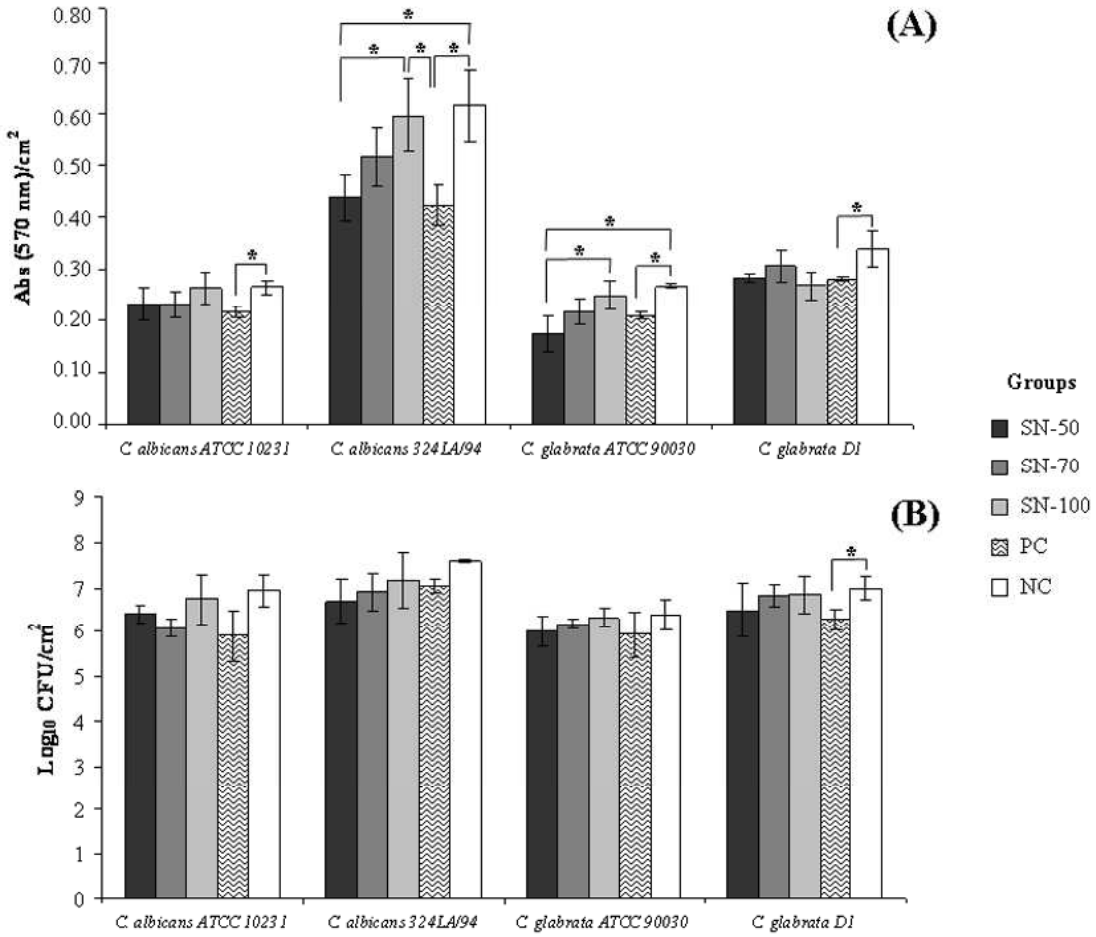

Figure 1. Mean values of the absorbance per cm^2 (A) obtained with crystal violet staining assay and means of the logarithm of colony forming units per cm^2 (B) for mature *Candida* biofilms (48 h) after 24 h of treatment with silver nanoparticles (SN) at 54 mg/L, which were pre-heated at 50 (SN-50), 70 (SN-70), and 100ºC (SN-100). PC = positive control (biofilms incubated with unheated SN). NC = negative control (biofilms incubated with RPMI 1640 medium without SN). Error bars represent the standard deviations of the means. (*) Denote $P < 0.05$, using ANOVA followed by Bonferroni test

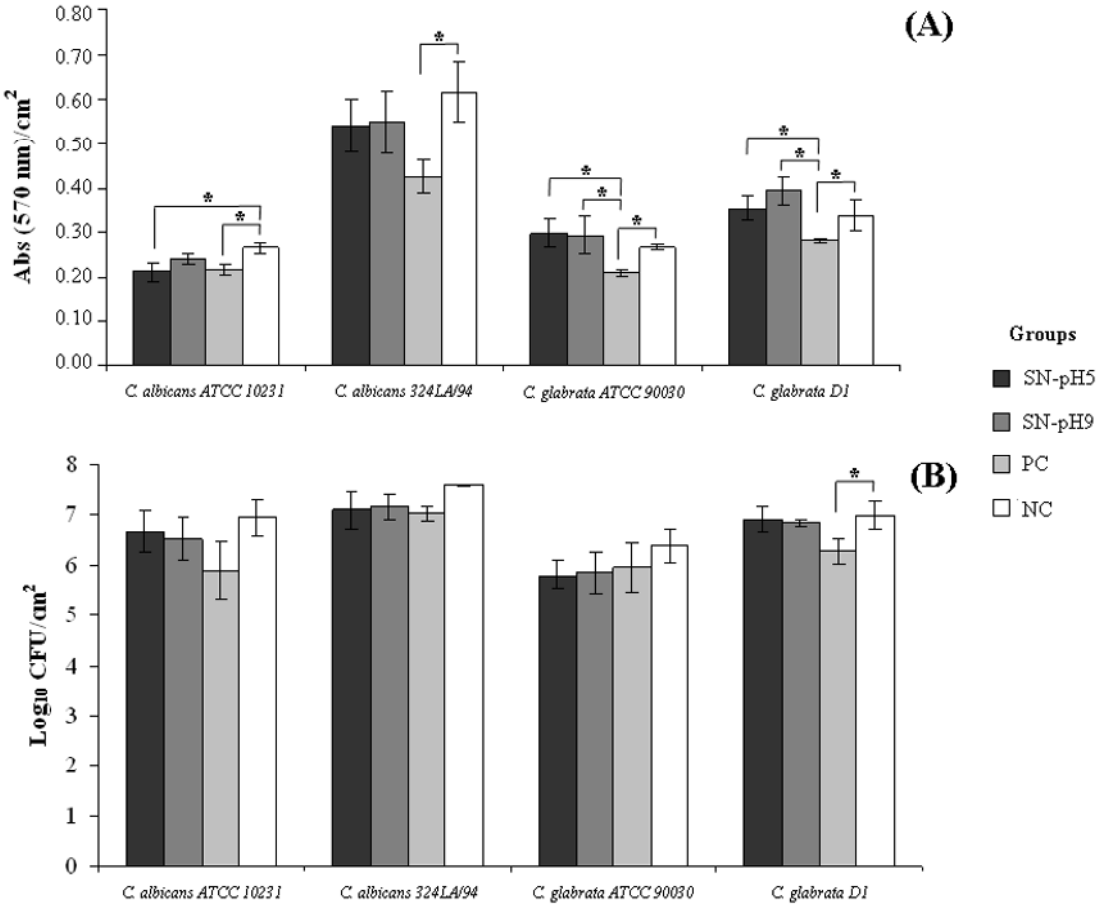

Figure 2. Mean values of the absorbance per cm^2 (A) obtained with crystal violet staining assay and means of the logarithm of colony forming units per cm^2 (B) for mature *Candida* biofilms (48 h) after 24 h of treatment with silver nanoparticles (SN) at 54 mg/L, which had the pH varied (during 2 h) to 5.0 (SN-pH5) and 9.0 (SN-pH9) before contact with biofilms. PC = positive control (biofilms incubated with SN without pH variation). NC = negative control (biofilms incubated with RPMI 1640 medium without SN). Error bars represent the standard deviations of the means. (*) Denote $P < 0.05$, using ANOVA followed by Bonferroni test

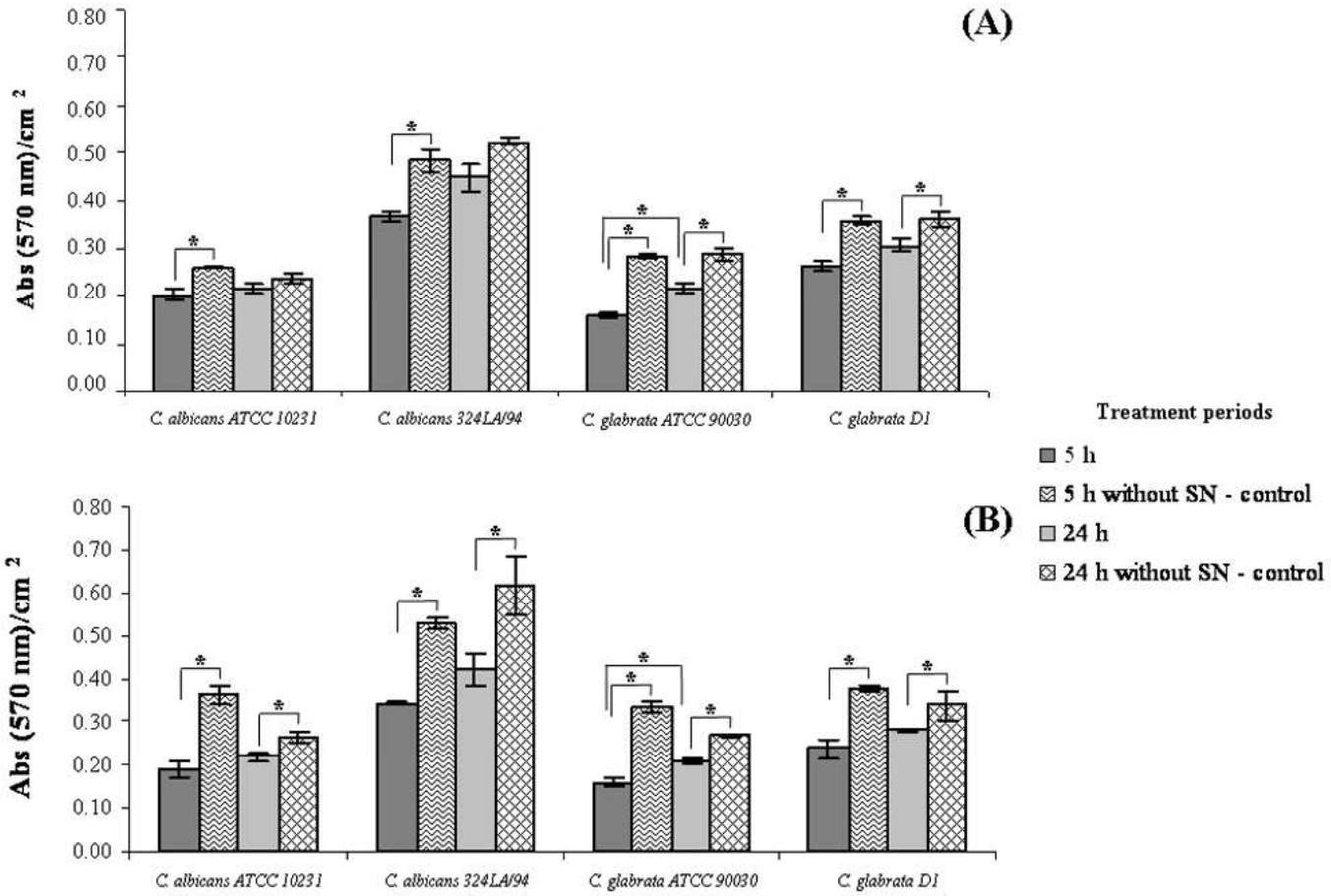

Figure 3. Absorbance values per cm^2 obtained with crystal violet staining assay for 24-h (A) and 48-h (B) *Candida* biofilms after different treatment periods with silver nanoparticles (SN) at 54 mg/L. Error bars indicate the standard deviations of the means. (*) Denote $P < 0.05$, using ANOVA followed by Bonferroni test

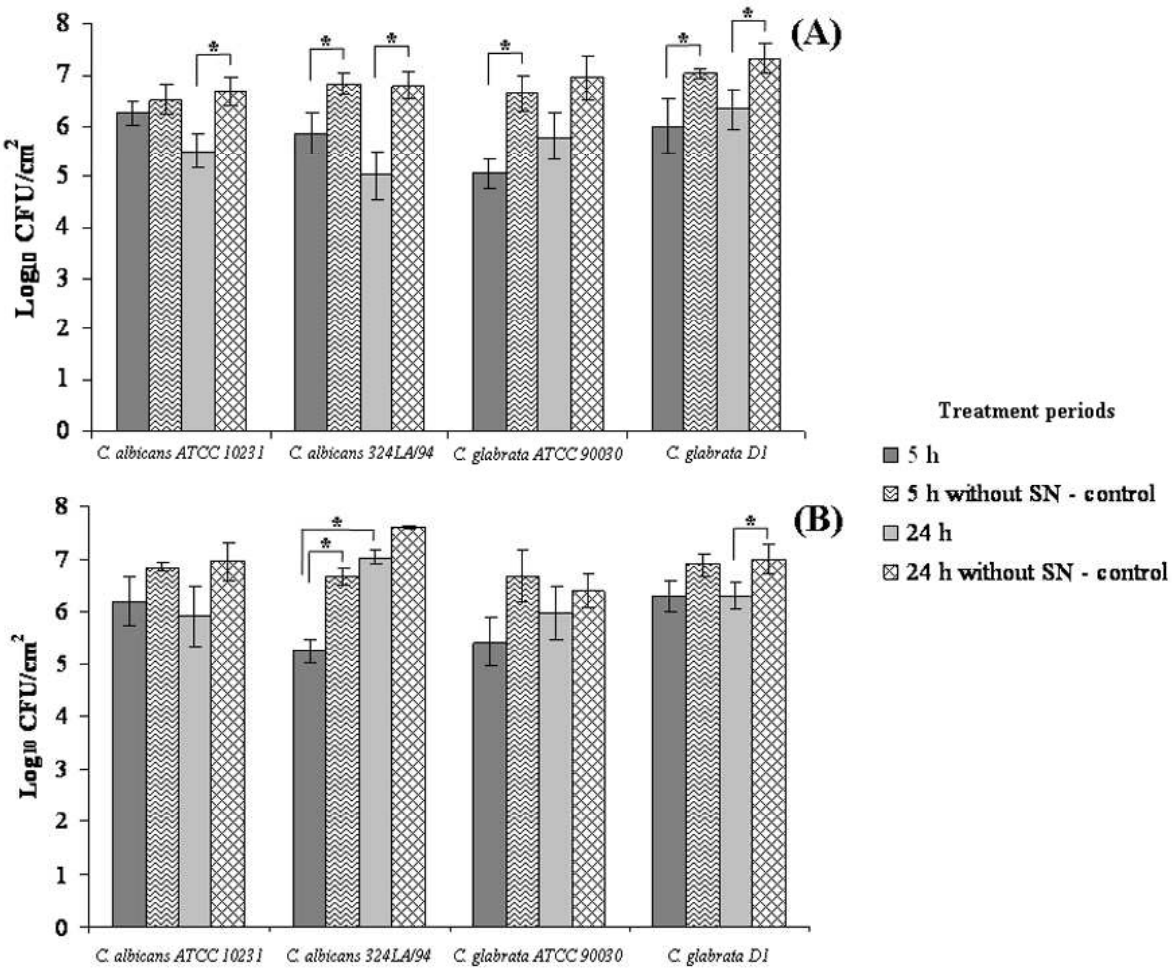

Figure 4. Logarithm of colony forming units per cm^2 obtained for 24-h (A) and 48-h (B) *Candida* biofilms after different treatment periods with silver nanoparticles (SN) at 54 mg/L. Error bars indicate the standard deviations of the means. (*) Denote $P < 0.05$, using ANOVA followed by Bonferroni test

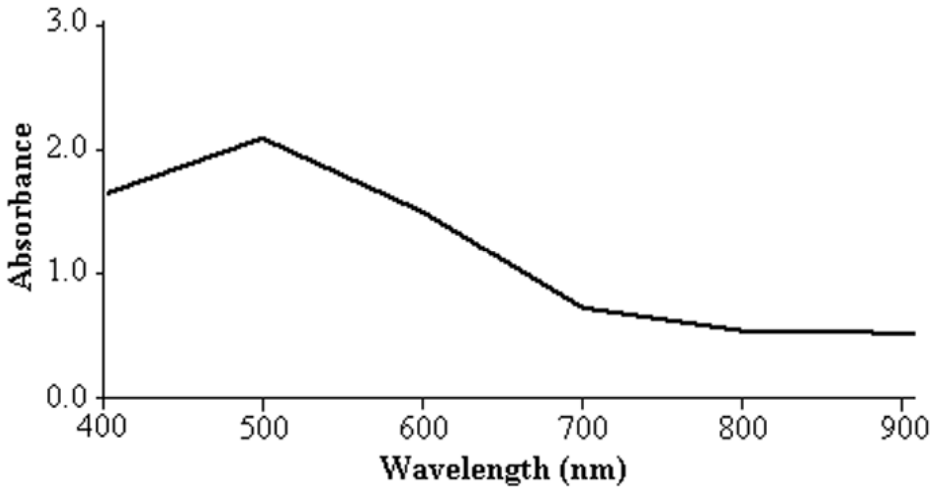

Figure 5. UV-visible spectra of silver nanoparticles (SN) colloidal suspension heated at 100ºC during 30 minutes

ANEXOS

Anexo A – Referências Gerais

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Anexo F – Normas do periódico *Journal of Basic Microbiology*

Edited By: Erika Kothe

Impact Factor: 1.266

ISI Journal Citation Reports © Ranking: 2011: 89/112 (Microbiology)

Online ISSN: 1521-4028

Instructions to authors (April 2010)

Authors are requested to follow these instructions carefully.
Manuscripts not prepared accordingly will be returned to authors and this will inevitably lead to a delay
in the editorial processing of the manuscript.

1. Aims and scope
2. General terms of publication
3. Online submission of manuscripts
4. Types of contributions
5. Organization of manuscripts
6. Guidelines for the preparation of electronic data
7. Revised manuscripts
8. Proofs and reprints
9. NIH authors
10. Reporting specific data

1 Aims and scope

The Journal of Basic Microbiology (JBM) publishes primary research papers on both procaryotic and eucaryotic microorganisms, including bacteria, archaea, fungi, algae, protozoans, phages, viruses, viroids and prions. Special emphasis is given to innovative results in basic research dealing with
– microbial interactions (pathogenic, mutualistic, environmental),
– ecology,
– physiology,
– genetics and cell biology/development,
– new methodologies, i.e., especially new imaging technologies (video-fluorescence microscopy, immunofluorescence of modern TEM applications) and novel molecular biology methods (PCR-based gene targeting or cassettes for cloning of GFP constructs).
Types of publication: original research papers, short communications, method papers, reviews and mini-reviews will be accepted for publication after strict peer-review.

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The manuscript should be accompanied by a letter of transmittal that should state why the paper is suitable for publication in the Journal and include the following FIVE statements:

1. All authors concur with the submission.
2. The work has not been published elsewhere, either completely, in part, or in another form.
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5. The manuscript does/does not contain human studies. If such studies are included, it should be stated that local Ethical Committee approval was received for the studies and that the informed consent of all participating subjects was obtained. Permission statements relating to points 4 and 5 should also be included in the text of the manuscript.

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Four types of scientific contributions are considered for publication:

1. Full Papers describing complete investigations. Unsolicited original papers should not exceed approximately 6.500 words; this includes references, figure legends and tables. Manuscripts may not have been published previously, except in the form of a preliminary communication.
2. Reviews will normally be invited by the Editor. Authors wishing to submit a review article should send a brief outline of its contents to the Editor-in-Chief before the manuscript is drafted.
3. Short Communications describing results that are brief, timely and/or of such importance that rapid publication is warranted. These manuscripts should bear the words "Short Communication" immediately above the title on the first page. They should also be subdivided into titled sections but should be written in a continuous style. Short communications should not exceed 2.500 words and contain no more than two figures and one table.
4. Method papers will describe the development of a novel method or an improvement or noteworthy modification of an already existing technique or platform used in biotechnology. These manuscripts should bear the words "Method Paper" immediately above the title on the first page. A method paper is a short (no more than two pages when published) description written in a continuous style with no more than two figures and one table.

5 Format and style of manuscripts
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Contents of first page of manuscript
The first page of the manuscript should contain only the following:
1. Title of the paper containing only the most important keywords pertaining to the subject matter. Only standard abbreviations should be used in the title.
2. Full names (including first name) of the authors and the name of their institute(s). If the publication originates from several institutes the affiliations of all authors should be clearly stated by using superscript numbers after the name and before the institute.
3. Keywords (3 to 5).
4. Name, title and full postal address of the author to whom all correspondence (including galley proofs) is to be sent. This should include fax and telephone numbers, and e-mail address.
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Abstract
The second and (if necessary) third page of the manuscript should contain the abstract only. This must be self-explanatory and intelligible without reference to the text. It should not exceed 200 words. Only standard abbreviations are allowed.

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"1 Introduction": containing a description of the problem under investigation and a brief survey of the existing literature on the subject.
"2 Materials and methods": for special materials and equipment, the manufacturer's name and if possible the location should be provided.
"3 Results"
"4 Discussion"
"5 References"
For Short Communications: Sections 3 and 4 may be combined and should then be followed by a short section entitled "Concluding remarks". Subdivisions of sections should be indicated by subheadings.

References
References, including those in tables and figure legends, should be numbered sequentially in the order in which they appear in the text. The numbers should be set in square brackets in the text i.e. [2,18]. References are to be collected in numerical order at the end of the manuscript under the heading "References", they should also be typed with double spacing throughout. Titles of journals should be abbreviated according to the practice of Chemical Abstracts. If necessary, cite unpublished or personal work in the text but do not include it in the reference list. The DOI for the reference should be included at the end of the reference, if no print reference is available. Abstracts and posters in meetings books must not be cited unless they are generally accessible. Please note that website addresses must not be included as a reference but should be inserted in the text directly after the data to which they refer. Responsibility for the accuracy of bibliographic references rests entirely with the author.
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[1] Liebeton, K., Eck, J., 2004. Identification and expression in *E. coli* of novel nitrile hydratases from the metagenome. Eng. Life Sci., **4**, 557–562.
[2] Hugo, G., Schmoekel, F.E., Weber, J.C., Jason C., et al., 2005. Bone repair with a form of BMP-2 engineered for incorporation into fibrin cell ingrowth matrices (p NA). Biotechnol. Bioeng., DOI:10.1002/bit.20168.
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[4] Chrisou, P., Klee, H. (Ed.), 2004. Handbook of Plant Biotechnology. John Wiley & Sons, New York.

Acknowledgements
Acknowledgements as well as information regarding funding sources should be provided on a separate page and will appear at the end of the text (before "5 References").

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Tables with suitable captions at the top and numbered with Arabic numerals should be collected at the end of the text on separate sheets (one page per table). Column headings should be kept as brief as possible and indicate units. Footnotes to tables should be indicated a), b), c) etc. and typed on the same page as the table.

Supplementary material
Extensive tables (more than 5 typewritten pages) should be published online as supplementary material. This material will not be typeset so authors should prepare this in the final form. Also for this reason there will be no galley proofs of this material. Supplementary material will be made freely available on the web (similar to the table of contents and the article abstracts). Authors are permitted to place this material on their homepages when they are setting up a link to the fulltext version of the article in Wiley InterScience. Further, other files may be submitted as supplementary material (e.g., animations, video sequences). Please contact the Editorial Office at jbmoffice@gmx.de for suitable file formats. All supplementary material will also undergo the peer review process. Thus, this material has to be submitted electronically along with the main body of the article. It is in the hands of the Editor-in-Chief to decide which part of the manuscript will be published as supplementary material.

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11 Reporting specific data

Chemical structures

Structures should be produced with the use of a drawing program such as ChemDraw. Structure drawing preferences are as follows:

• As drawing settings select:
chain angle 120°
bond spacing 18% of width
fixed length 14.4 points (0.508 cm, 0.2 in.)
bold width 2.0 points (0.071 cm, 0.0278 in.)
line width 0.6 point (0.021 cm, 0.0084 in.)
margin width 1.6 points (0.056 cm, 0.0222 in.)
• hash spacing 2.5 points (0.088 cm, 0.0347 in.)
• As text setting select: font, Arial or Helvetica; size, 10 pt.
• Under the preferences choose: units, points; tolerances, 3 pixels.
• Under page setup choose: paper, US Letter; scale, 100%.
• Using the ChemDraw ruler or appropriate margin settings, create structure blocks, schemes, and equations having maximum widths of 11.3 cm (one-column format) or 23.6 cm (two column format). Note: if the foregoing preferences are selected as cm values, the ChemDraw ruler is calibrated in cm. Also note that a standard sheet of paper is only 21.6 cm wide, so all graphics submitted in two column format must be prepared and printed in landscape mode.
• Use boldface type for compound numbers but not for atom labels or captions.
• Authors using other drawing packages should, as far as possible, modify their program's parameters to reflect the above guidelines.

Physical and other data

It is important that novel compounds, either synthetic or isolated/produced from natural sources, be characterized completely and unambiguously. Supporting data normally include physical form, melting point (if solid), UV/IR spectra if appropriate, 1H and 13C NMR, mass spectral data, and optical rotations or CD information (when compounds have chiral centers). Reports on flavor constituents should conform to the recommendations made by the International Organization of the Flavor Industry (IOFI). Thus, any identification of a substance has to be done by the latest form of available analytical techniques. In general, any particular substance must have its identity confirmed by at least two methods; that means, in practice, comparison of chromatographic and spectroscopic data (which may include GC, MS, IR, and NMR) with those of an authentic sample. If only one method has been applied, the identification has to be labeled as "tentative": This is also valid in case of identification performed only by comparison of literature data.

Equations should be numbered consecutively and referred to in the text; e.g. "defined as in Eq. (1)".

Physical data should be quoted with decimal points (e.g. 25.8 J/K × mol), and arranged as follows where possible — but in any event in the same order within the manuscript (when measurement conditions remain unchanged they need only be mentioned once, for instance in the column headings): m.p./b.p. 20 °C; [α]20 D = –13.5 (*c* = 0.2 in acetone); 1H NMR (200 MHz, [D_8]THF, 25 °C, TMS): δ = 1.3 (q, ^3J(H,H) = 8 Hz, 2 H; CH_2), 0.9 ppm (t, ^3J(H,H) = 8 Hz, 3 H; CH_3);

IR(Nujol): $\nu\sim$ = 1790 cm^{-1} (C=O); UV/VIS (*n*-hexane): $\lambda max(\varepsilon)$ = 320 (5000), 270 nm (12 000); MS (70 eV): *m*/*z* (%): 108 (20) [M+], 107 (60) [M+–H], 91 (100) [C_7H_7 +]. Plane angles in products of units can have either ° or deg as the unit.
Nomenclature, symbols, and units: The rules and recommendations of the International Union of Pure and Applied Chemistry (IUPAC), the International Union of Biochemistry (IUB), and the International Union of Pure and Applied Physics (IUPAP) should be adhered to.
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Proteins Protein sequences, which have been determined by direct sequencing of the protein, must be submitted to Swiss-Prot at the EMBL Outstation – The

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Anexo G – Normas do periódico *Journal of Antimicrobial Chemotherapy*

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All articles should include a title page comprising: article title; author names and their affiliations (each affiliation address must be given separately and in full); telephone, fax and e-mail contact details for the corresponding author; a short running title; and 3-5 keywords (very general terms such as 'bacteria' and 'human' and terms already present in the title should be avoided, as should non-standard abbreviations). In addition, all articles must include a Funding section (if reporting original research) and a Transparency declarations section.

Original articles and Brief reports must have a structured synopsis. The headings for the structured synopsis are as follows: Objectives, Patients and methods (or Methods), Results, and Conclusions.

Original articles. There is no length limit for this format; however, papers must be written as concisely as possible. Original articles are divided into the following sections: Synopsis (250 words maximum), Introduction, Materials (or Patients) and methods, Results, Discussion, Acknowledgements, Funding, Transparency declarations and References. Repetition of content between sections must be avoided. A combined Results and Discussion section is acceptable.

Brief reports. These should have the same format as Original articles, but should have no more than two figures/tables, should have a maximum of 20 references and should not exceed 1500 words of text.

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Case reports. JAC will publish Case reports that are of sufficient calibre and potential importance, and they should be submitted in the form of Correspondence (see above). Please note that patient anonymity MUST be preserved in Case reports (see the later section on Ethics approval and patient consent/privacy).

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consider unsolicited reviews, but authors are encouraged to consult the Editor-in-Chief in advance of writing to avoid duplicating commissioned material.

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Please note that on publication all Original articles and Brief reports, as well as Antimicrobial practice papers, will be published under the heading of Original research so that articles on similar topics can be grouped together when assigned to an issue. In addition each piece of Correspondence will be published as either a Research letter or a Letter to the Editor.

Peer review

After preliminary examination of the submission by Editorial Office staff to check that all the necessary elements are present, the paper is passed to the Editor-in-Chief. The Editor-in-Chief then assigns the paper to an appropriate Senior Editor. The Senior Editor is then responsible for selecting an Editor to handle the article. Articles can be rejected immediately by the Editor-in-Chief, a Senior Editor or an Editor without further peer review. The assigned Editor is responsible for selecting referees and obtaining referee reports.

The usual number of referees is two, however, the Editors reserve the right to make a decision on a paper on the basis of one referee report, or seek the opinion of more than two referees if they judge this to be necessary or desirable. Leading articles and Correspondence are not routinely sent for external refereeing, but the Editor-in-Chief, Senior Editors and Editors reserve the right to seek the opinion of one or more external referees if they judge this to be necessary or desirable. Senior Editors, Editors and referees are asked to consider whether they have any conflicts of interest when they are assigned a paper, and if necessary to decline to handle the paper. See the section ‘Conflicts of interest’ for more information on this subject.

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In-press and submitted papers that are important for the review of a paper MUST be uploaded when the paper is submitted and referred to in the covering letter that accompanies the submission. Authors should be aware of the issues of redundant/duplicate publication. For further information, please see the following Editorial:

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Authorship

The authorship of the paper should be confined to those who have made a significant contribution to the design and execution of the work described. In the case of clinical trials/randomized control trials it is compulsory for the contribution of each author to be clearly stated in the Transparency declarations section, after the information on conflicts of interest. Authors of other types of article may indicate the contribution made by each author if they wish.

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Many large collaborative studies (frequently resistance surveys) are organized under a group name that represents all of the participants. *JAC* will not accept a group name as an 'author' of an article. All articles must have at least one named individual as author. Authors of large collaborative studies should list the author(s) of the article and follow this with 'on behalf of the GROUP NAME'. The names of all of the participants should then be listed in the Acknowledgements section.

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Professional medical writers and other forms of writing assistance have an important role to play in the clear communication of scientific results. However, unless this role is openly explained and acknowledged unfounded suspicions about this role will continue. *JAC* encourages the open and precise description of any such assistance received by authors in relation to any article. It is possible that writers may qualify for authorship of a manuscript, we recommend that authors review the ICMJE criteria for authorship before submission

(http://www.icmje.org/#author).The precise role of the writer or service in the origin or preparation of the manuscript must be declared in the Transparency declarations section; we recommend that the name of the writer (and their agency where applicable) or the service is provided. If this support was funded, the source must be declared in the Funding section.

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Authors must indicate in the Methods whether the research was conducted in accordance with the Declaration of Helsinki and national and institutional standards. If approval was obtained from an Ethics Committee the authors should indicate this, as well as any approval/reference number. Written informed consent must be obtained from study participants and the existence of this consent must be stated in the article.

Patient privacy. Patients have a right to privacy. Any information that might result in identification of individuals must be omitted, especially if it is not directly clinically relevant. Patient age, sex, admission dates and co-morbidities should be removed as far as possible. If it is possible that a patient could be identified, the

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Authors must list any internal funding. If no specific funding has been received then this should be clearly stated; equally if data have been generated as part of the routine work of an organization, this too should be stated. Ongoing financial support for any of the authors should also be included under the Funding heading.

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- The sentence should begin: 'This work was supported by …'
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- Where individuals need to be specified for certain sources of funding the following text should be added after the relevant agency or grant number 'to (author initials)'.

An example is given here: 'This work was supported by the National Institutes of Health (P50 CA098252 and CA118790 to R. B. S. R.) and the Alcohol & Education Research Council (HFY GR667789).

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In the case of clinical trials/randomized control trials it is compulsory for the contribution of each author to be clearly stated in the Transparency declarations section, after the information on conflicts of interest. Authors of other types of article may indicate the contribution made by each author if they wish.

Other useful information

In some instances (often when the authors themselves have no interests to declare) it may be helpful to readers as background information to give brief details of organizations that do have an interest but do not appear elsewhere in the article, for example 'Fantastazole is owned by Wonder Pharmaceuticals'.

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We will energetically pursue accusations of misconduct directed at authors, Editors or referees and have a number of sanctions at our disposal including the option to inform employers about accusations and ask them to mount their own internal investigations. Accusations should not be made lightly or in the absence of the likelihood of supporting evidence being obtainable. The Journal may take the view that accusations are malicious if supporting evidence cannot be found and may direct sanctions against accusers in such cases. Any accusation of misconduct should be addressed to the Editor-in-Chief (unless it involves the Editor-in-Chief, in which case it should be directed to the Chairman of the Advisory Board). *JAC* is a member of COPE and will follow its guidelines on the handling of investigations into research misconduct.

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Authors must register their trials in one of the databases dedicated to registration of trials. In addition, authors must state the database and provide the unique registration number – both in the abstract and in the main body of the paper.

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Reporting standards

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There is a wide range of reporting guidelines, each specific for different types of study. Some of those for study types that are frequent in *JAC* are mentioned specifically below. Authors should consult the EQUATOR network website (http://www.equator-network.org/) for links to the latest versions of guidelines, which are organized by the study type.

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International Society of Pharmacoeconomics and Outcomes Research (ISPOR) Checklist for retrospective database studies, which can be accessed at: http://www.ispor.org/workpaper/healthscience/ret_dbTFR0203.asp
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JOURNAL STYLE

General
In addition to reading the information provided here, authors should consult a recent issue of the Journal for the layout and conventions used.

The past tense should be used throughout for description of the results of the paper, the present tense should be used when referring to previously established and generally accepted results.

Where possible SI units should be used.

Please ensure that characters with a similar appearance are consistent throughout the document and not from different Unicode sub ranges as with the Greek Delta.

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Spelling
British spelling should be used. Spelling should follow that of the Oxford Dictionary for Scientific Writers and Editors and where this gives no guidance the Concise Oxford Dictionary. Spelling of drug names should conform with that given in the latest edition of the British National Formulary (published by the British Medical Association and the Royal Pharmaceutical Society of Great Britain and available online at http://www.bnf.org/bnf), but please note that JAC will continue to use methicillin (not meticillin).

Abbreviations
Non-standard abbreviations should be defined at the first occurrence and introduced only where multiple use is made. See here for abbreviations that may be used without definition, as well as antimicrobial abbreviations (which may be used in Tables and Figures).

Dosage and routes of administration

Dosage frequencies should be given in full in English at each occurrence. Abbreviations are not permitted. Routes of administration other than intramuscular (im) and intravenous (iv), which may be abbreviated after definition, should be given in full in English.

MICs
Please note that all MIC data in JAC must be expressed in terms of mg/L (not μg/mL).

Bacterial nomenclature
When genus and species are given together use a capital letter for the genus and a lowercase letter for the species and italicize both e.g. Staphylococcus aureus. After the initial use in the text of the full name of an organism the generic name should then be abbreviated to the initial letter, e.g. E. coli.

When the genus is used as a noun or adjective use lowercase roman unless the genus is specifically referred to e.g. 'staphylococci and streptococci' but 'organisms of the genera Staphylococcus and Streptococcus'.

The name of an order has an initial capital but is not italicized, e.g. Enterobacteriaceae. For genera in the plural, use lowercase roman, e.g. salmonellae.

When the species is used alone use lowercase e.g. viridans streptococci. For trivial names, use lowercase roman e.g. meningococcus.

Authors should use bacterial names present in the Approved List of Bacterial Names, Amended Edition (1989), Skermanm, V.B.D., McGowan, V. & Sneath, P.H.A., Eds, ASM Press, Washington, DC, USA (ISBN 1-55581-014-4), with subsequent alterations validly published by announcement in Validation Lists of the International Journal of Systematic and Environmental Microbiology (formally the International Journal of Systematic Bacteriology). A full list of validly published bacterial names is given at http://www.bacterio.cict.fr/allnames.html

Genetic and amino acid nomenclature
Bacterial genetics. Genotype designations are indicated with italic lowercase three-letter locus codes (e.g. par, his, ara). If several loci are involved in a related function the individual loci are designated by the addition of an uppercase italic letter to the locus code (parC, ompF).

Phenotype designations (for example the protein product of a bacterial gene) are given in roman type with an initial capital letter (OmpF, LacZ).

Erythromycin gene nomenclature should follow that described in: Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J & Seppala H. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob Agents Chemother 1999; 43: 2823-30.

Yeast genetics. Wild-type alleles are all uppercase and italicized (LEU2), mutant alleles are all lowercase and italicized (leu2), and gene products are capitalized on the first letter and are not italicized (Leu2).

General. Authors should ensure that they confine discussion of changes in amino acid sequence to the context of the protein (e.g. OmpF) and nucleotide changes to the context of the gene (e.g. ompF). Please also be aware of the difference between a mutant (a strain with one or more mutations) and a mutation (a change in the sequence of the genetic material).

Amino acids. The full residue names or three-letter abbreviations are preferred in the text (e.g. a methionine residue at position 184 should be symbolized Met-184). The single letter codes may be used in figures. Amino acid changes should be designated Met-184→Val or M184V.

When comparing nucleotide or amino acid sequences authors should exercise care in the use of the term homology. Homology should only be used when a common evolutionary origin is being implied; it is incorrect to give a percentage homology between two sequences. The wing of a bird and the human arm are homologous structures (they are believed to have a common evolutionary origin), homology cannot be quantified. For sequence comparison authors should use the terms identity and similarity. Sometimes 'equivalent' or 'counterpart' is more appropriate than 'homologue'.

Beta-lactamase nomenclature
Authors submitting articles reporting the identification of new beta-lactamases must provide evidence that they have contacted the relevant clearinghouse (http://www.lahey.org/Studies/) to deposit the new sequence data and receive a unique designation for the new enzyme.

Macrolide-lincosamide-streptogramin resistance determinant nomenclature
Nomenclature for macrolide-lincosamide-streptogramin resistance determinants should follow the structure suggested by: Roberts MC, Sutcliffe J, Courvalin P et al. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B antibiotic resistance determinants. Antimicrob Agents Chemother 1999; 43: 2823-30. A new gene must have ≤79% amino acid identity with all previously characterized MLS genes before receiving a new unique name. Adding subscripts or superscripts to established genes is not acceptable. See: http://faculty.washington.edu/marilynr/. Before submitting a sequence to GenBank or submitting a manuscript for publication, please contact Professor Marilyn Roberts (marilynr@u.washington.edu). Once a new name has been assigned you must indicate in your article that you have received approval by the nomenclature centre for the new gene name.

Tetracycline resistance determinant nomenclature
Nomenclature for tetracycline resistance determinants should follow that suggested by: Levy SB, McMurry LM, Barbosa TM et al. Nomenclature for new tetracycline resistance determinants. Antimicrob Agents Chemother 1999; 43: 1523-4. A new gene must have ≤79% amino acid identity with all previously characterized tet genes before receiving a new unique name. Adding subscripts or superscripts to established genes is not acceptable. See: http://faculty.washington.edu/marilynr/. The Levy Group is responsible for

coordinating the naming of new tet genes and before submitting a sequence to GenBank or submitting a manuscript for publication, please contact Laura McMurry (laura.mcmurry@tufts.edu). Once a new name has been assigned you must indicate in your article that you have received approval by the nomenclature centre for the new gene name.

qnr gene/allele nomenclature
Authors submitting articles reporting the identification of new qnr genes or alleles must provide evidence that they have contacted the relevant clearinghouse (http://www.lahey.org/qnrStudies/) to deposit the new sequence data and receive a unique designation. Authors should consult Jacoby G, Cattoir V, Hooper D et al. qnr gene nomenclature. Antimicrob Agents Chemother 2008; 52: 2297-9.

FICI data
Fractional inhibitory concentration index (FICI) experiments are performed in order to study drug interactions and they must be interpreted in the following way:

FICI<=0.5 = synergy

FICI>4.0 = antagonism

FICI>0.5-4 = no interaction

For further information please see the following Editorial:

Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother 2003; 52: 1.

Microarray data
Authors of articles containing microarray data must ensure that the full datasets are lodged with an appropriate publicly available online database (the data must not be supplied for publication as Supplementary data alongside the article). The data should be supplied with the submitted article if they are not already publicly available. The name of the database and the accession numbers should be provided in the article. Authors must ensure that their data are available for public scrutiny from the online publication date of their article at the latest.
Chemistry
General nomenclature. The IUPAC recommendations on chemical nomenclature should be followed (IUPAC Compendium of Chemical Terminology (1987, ISBN 0 632 01767 8, Blackwell Scientific Publications, Oxford). All chemical names are run together except those of acids, acetals, esters, ethers, glycosides, ketones and salts, which are printed as separate words; hyphens are used to separate numbers, Greek letters and some configurational prefixes, e.g. p-nitrophenol. Italics are used for certain prefixes, e.g. cis-, trans- and N. Small capitals are used for dextro- and laevo- prefixes, e.g. L-glutamine.

Drugs. Spelling of drug names should conform with that given in the latest edition of the British National Formulary. Chemical or generic names of drugs should be

used; trade names may be referred to once only upon first use of the generic or chemical name. The content of proprietary formulations should be given if relevant. Generic names should not be abbreviated in the text; abbreviations may be used in Tables if there is limited space. If compounds are referred to by code name or company number either the structure or a reference to a paper illustrating the structure must be given, any previous code names or designations should be given on first use.

Supplier locations are required for all smaller/local suppliers.

References
Authors are responsible for the accuracy of all references, which must be checked against the original material. Reference citations should be restricted to those that are essential for introducing the purpose and context of the paper, describing methods that are not given in detail, and for discussing the results and any relevant issues raised by them. Authors are responsible for ensuring that references are quoted accurately and not taken out of context. References must not be cited in the synopsis.

Where possible authors should avoid citing conference abstracts or posters (partly because they are not peer reviewed and also because they often report interim findings and the final published studies can often come to substantially different conclusions) and authors MUST NOT cite abstracts that are more than 2 years old without excellent justification for doing so. In addition, abstracts must only be cited if they appear in published abstract books, journal supplements or in a permanent online archive.

References should be cited in the text using sequential numbers. Superscript numbers should be used and should be placed after any punctuation. When referring to several references, separate individual numerals by a comma or a hyphen for a range greater than two references. For instance: This was first discovered by Jones,1 and later confirmed by several other groups of investigators.2,3,5-7

Papers accepted for publication, but not yet published, may be included in the reference list; they should be listed as 'in press', with the name of the journal and the likely year of publication. Submitted work should be quoted as 'unpublished results'. Personal communications and unpublished results, which are permitted in the text only, must include the initials and surnames of all the workers involved; for the former citation, the person’s affiliation must be stated, e.g. ‘(J. Bloggs, NIH, personal communication)’, and documentary evidence (an e-mail will suffice) from the person quoted, showing their agreement to be so quoted, must be provided (the agreement must include the exact wording that appears in the paper).

All references should be listed numerically at the end of the text. Each reference should be preceded by a number (not superscript) followed by a full stop. Please

see the following examples. Failure to conform to Journal style will result in the manuscript being returned to authors.

Examples

Journal reference (<= three authors)
Sanschagrin F, Levesque RC. A specific peptide inhibitor of the class B metallo-B-lactamase L-1 from Stenotrophomonas maltophilia identified using phage display. J Antimicrob Chemother 2005; 55: 252-5.

Journal reference (> three authors)
Williams I, Gabriel G, Cohen H et al. Zidovudine-the first year of experience. J Infect 1989; 18 Suppl 1: 23-31.

Whole book
Long HC, Blatt MA, Higgins MC et al. Medical Decision Making. Boston: Butterworth-Heinemann, 1997.

Book chapter
Manners T, Jones R, Riley M. Relationship of overweight to haitus hernia and reflux oesophagitis. In: Newman W, ed. The Obesity Conundrum. Amsterdam: Elsevier Science, 1997; 352-74.

NCCLS/CLSI methods
National Committee for Clinical Laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically—Sixth Edition: Approved Standard M7-A6. NCCLS, Wayne, PA, USA, 2003.

Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Fifteenth Informational Supplement M100-S15. CLSI, Wayne, PA, USA, 2005.

Meeting abstract
Hou Y, Qiu Y, Vo NH et al. 23-O derivatives of OMT: highly active against H. influenzae. In: Abstracts of the Forty-third Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, 2003. Abstract F-1187, p. 242. American Society for Microbiology, Washington, DC, USA.
Online material
References to online material should be given in the reference list. It is important that the date you last accessed the site be included and that you check the site at the proof stage to ensure that the material is still available. Please note that URLs for the suppliers of materials must not be given in either the text or the references. The Journal does not accept any responsibility for the content of web pages cited.

NORM/NORM-VET 2000. Consumption of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. http://www.vetinst.no/Arkiv/Zoonosesenteret/NORM_VET_2000.pdf (3 May 2005, date last accessed).

For online-only journals the following reference format should be used:

Health Protection Agency. The third year of regional and national analyses of the Department of Health's mandatory MRSA surveillance scheme in England: April 2001-March 2004. CDR Wkly 2004; 14(29). http://www.hpa.org.uk/cdr/archives/2004/cdr2904.pdf (28 June 2005, date last accessed).

Tables

These should be employed sparingly and should be generally comprehensible without reference to the text. Each table should be supplied on a separate sheet and numbered consecutively using Arabic numerals in the order they are referred to in the text. Each must have a brief descriptive heading. Column headings must clearly explain the content of the column and indicate any units used. Footnotes should be kept to a minimum.

Tables must be created using the Table function in Word; they must not be inserted as images. Each data item should occupy a single cell and return characters should not be used within any Table. JAC reserves the right to move complicated Tables to online-only Supplementary data.

Figures

These must be employed sparingly to demonstrate important specific points. Figures should be numbered using Arabic numerals in the order in which they are referred to in the text. In figure LEGENDS, symbols should be described in words (e.g. filled circles, open squares etc.).

Wherever possible, figures should be two-dimensional. Authors should NOT supply 'three-dimensional' figures unless this is actually necessary to represent the data.

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Figures should be sized to fit a single column of the Journal where possible (88 mm) or a double column if necessary (180 mm). The preferred font for lettering is

Times; lettering should have an upper case height of 2 mm and a lower case height of 1 mm at publication size (corresponding to point size 8). Line thickness should be set at 0.5 points. Shading used on line drawings should be clear and distinctive; shades of grey and heavy stippling do not reproduce well. Lines and symbols should be drawn boldly enough to withstand reduction. The preferred symbols are filled circles, open circles, filled squares, open squares, filled triangles and open triangles, and should be no smaller than 1 mm (height/diameter) at publication size. Part labels should be lower case letters within parentheses, e.g. (a), (b), (c) etc.