

The effect of silver nanoparticles and nystatin on mixed biofilms of *Candida glabrata* and *Candida albicans* on acrylic

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The aim of this study was to compare biofilm formation by *Candida glabrata* and *Candida albicans* on acrylic, either individually or when combined (single and dual species) and then examine the antimicrobial effects of silver nanoparticles and nystatin on these biofilms. Candidal adhesion and biofilm assays were performed on acrylic surface in the presence of artificial saliva (AS) for 2 h and 48 h, respectively. *Candida glabrata* and *C. albicans* adherence was determined by the number of colony forming units (CFUs) recovered from the biofilms on CHROMagar® *Candida*. In addition, crystal violet (CV) staining was used as an indicator of biofilm biomass and to quantify biofilm formation ability. Pre-formed biofilms were treated either with silver nanoparticles or nystatin and the effect of these agents on the biofilms was evaluated after 24 h. Results showed that both species adhered to and formed biofilms on acrylic surfaces. A significantly ($P < 0.05$) higher number of CFUs was evident in *C. glabrata* biofilms compared with those formed by *C. albicans*. Comparing single and dual species biofilms, equivalent CFU numbers were evident for the individual species. Both silver nanoparticles and nystatin reduced biofilm biomass and the CFUs of single and dual species biofilms ($P < 0.05$). Silver nanoparticles had a significantly ($P < 0.05$) greater effect on reducing *C. glabrata* biofilm biomass compared with *C. albicans*. Similarly, nystatin was more effective in reducing the number of CFUs of dual species biofilms compared with those of single species ($P < 0.05$). In summary, *C. glabrata* and *C. albicans* can co-exist in biofilms without apparent antagonism, and both silver nanoparticles and nystatin exhibit inhibitory effects on biofilms of these species.

Keywords Oral candidosis, co-colonization, biofilms, silver nanoparticles, nystatin

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Introduction

Oral candidosis is an opportunistic infection of the oral cavity caused by *Candida* species. Over recent decades, oral candidosis has increased in incidence, which is largely attributed to the increase in HIV-infections and the AIDS epidemic [1]. *Candida albicans* generally accounts for an estimated 50% of all candidosis cases [1,2]. However,

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importantly the incidence of *C. glabrata* as an oral pathogen in humans has also increased in recent years [3–6]. This is evident both in terms of single-species infection, as well as mixed infections most frequently with *C. albicans* [3,7–8]. The significance of this is that *C. glabrata* is often less susceptible to frequently used antifungal agents compared with *C. albicans* which makes treatment more problematic. Furthermore, in HIV and cancer patients, *C. glabrata* infections also tend to be more severe [9,10].

An important feature of *Candida* species in causing infection is their ability to adhere and form biofilms on host surfaces and implanted medical devices [11]. Furthermore, the increased use of oral medical devices such as complete or partial acrylic dentures has been associated with promoting biofilm formation and thus the development of oral candidosis [12,13]. In addition, increased tolerance of biofilm cells to administered antimicrobial agents is widely recognized [1]. As a result, there is a need to develop new agents that exhibit both a broad spectrum of antifungal activity and are used in combating biofilms. In this respect, the potential of silver nanoparticles (SN) as antimicrobials against a wide range of bacteria has been established [12–14], although little is known about their effects against *Candida* species [15].

In this present study, single and dual species colonization of acrylic by *C. glabrata* and *C. albicans* was examined to assess antagonistic and synergistic effects. In addition, the ability of both nystatin and SN on inhibiting the generated biofilms was determined.

Material and methods

Organisms and growth conditions

Candida albicans 324LA/94 and *C. glabrata* D1 were oral isolates obtained from the culture collection of the School of Dentistry at Cardiff University (Cardiff, UK) and the Biofilm Group of the Centre of Biological Engineering, University of Minho (Braga, Portugal), respectively. The identity of both isolates was presumptively determined using CHROMagar® *Candida* (CHROMagar, Paris, France) and definitive identification was achieved by PCR sequencing, employing specific primers (ITS1 and ITS4) targeting the fungal 5.8 S ribosomal RNA subunit [16].

Isolates were grown aerobically on Sabouraud dextrose agar (SDA; Liofilchem, Italy) at 37°C for 48 h. Selected colonies were then inoculated into Sabouraud dextrose broth (SDB; Liofilchem, Italy) and incubated at 37°C for 18 h under gentle agitation (120 rpm/min). After incubation, cells were harvested by centrifugation at 3000 g for 5 min at 4°C and washed twice with phosphate buffered saline (PBS; pH 7). Cell pellets were then suspended in artificial saliva (AS; pH 6.8). The composition of AS, as

previously described [18] with slight modification, was 2 g/l of yeast extract (Liofilchem, Italy), 5 g/l of peptone (Liofilchem, Italy), 2 g/l glucose (AppliChem, Germany), 1 g/l mucin (Sigma-Aldrich, USA), 0.35 g/l NaCl (AppliChem, Germany), 0.2 g/l CaCl₂ (Riedel-de Haën, Germany) and 0.2 g/l KCl (Pronalab, Portugal). The cell density in AS was adjusted to 2×10^7 cells/ml after enumeration using a Neubauer haemocytometer.

Preparation of acrylic resin specimens

For standardization of acrylic resin specimens, stainless steel patterns (10 × 10 × 3 mm) were imprinted in dental stone molds prepared in metallic flasks. After the setting of the stone, the flasks were separated, the stainless steel patterns removed, and the stone molds cleansed. Thermopolymerized acrylic resin (MEGDENTAL GmbH, Germany) was mixed, packed and pressed directly on to the dental stone molds, and polymerized according to the manufacturer's instructions. After polymerization and bench cooling at room temperature, the specimens were deflasked and finished using silicon carbide papers. Prior to each experiment, each acrylic square (1 × 1 cm²) was autoclaved at 121°C for 15 min.

Adhesion and biofilm formation

For single species adhesion and biofilm assays, acrylic squares were placed in 24-well tissue culture plates (Orange Scientific, Belgium), 500 µl of the standardized cell suspension (2×10^7 cells/ml in AS) was added and the volume adjusted to 1 ml with AS. In the case of dual species assays, 500 µl (2×10^7 cells/ml) of each species was added to each well. For controls, acrylic surfaces were similarly processed with AS devoid of *Candida*. Tissue culture plates were incubated at 37°C in an orbital shaker (120 rev/min) for 2 h in adhesion experiments and for 48 h for biofilm formation studies. In the case of biofilms, 500 µl of AS was removed and an equal volume of fresh AS added after 24 h.

Adhesion and biofilm quantification

Quantification of adhered and biofilm cells

The number of adherent cells and those present in biofilms was determined by enumerating colony-forming units (CFUs). Briefly, acrylic squares were washed twice with PBS (0.1 M; pH 7) to remove loosely attached cells. Squares of acrylic were then immersed in 1 ml of PBS (0.1 M; pH 7) and sonicated (Ultrasonic Processor, Cole-Parmer) for 30 s at 40 W. Complete removal of adherent cells was confirmed by crystal violet (CV) staining of the

treated acrylic square (as described below). The 1 ml suspensions were then vigorously vortex for 5 min and serial decimal dilutions (in PBS) inoculated on to CHROMagar® *Candida* (CHROMagar, Paris, France) plates which were incubated for 48 h at 37°C. After incubation, resulting colonies were counted and transformed to Log₁₀ CFU and standardized according to the area of acrylic squares (Log/cm²).

Quantification of total biofilm biomass

Total biofilm biomass was measured using a crystal violet (CV) staining method as previously described [17]. Briefly, after 48 h of biofilm formation, the medium was aspirated and non-adherent cells removed by washing the acrylic squares with 1 ml of PBS (0.1 M, pH 7). *Candida* biofilms were then fixed with 1 ml of 100% (v/v) methanol, which was removed after 15 min. The acrylic squares were allowed to dry at room temperature, 1 ml of CV (1% v/v) was then added to each well and incubated for 5 min. Acrylic squares were gently washed with sterile water and 1 ml of acetic acid (33% v/v) added to extract the CV from the biofilms. Optical density of the eluted solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa) at 570 nm. The final absorbance was standardized according to the area of acrylic squares (Abs/cm²). The experiments were performed in triplicate and repeated in a minimum of three independent assays.

Scanning electron microscopy

Acrylic squares were also analyzed by scanning electron microscopy (SEM) to examine the adhered cells and the biofilm cells structure. Thus, after 2 h and 48 h of incubation, for both single and dual species experiments, the AS was aspirated and the non-adherent cells removed by washing twice the squares with 1 ml of PBS (0.1 M, pH 7). They were then dehydrated through an alcohol series (70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air dried for 20 min. The acrylic squares were then maintained in a desiccator and prior to observation mounted on to aluminum stubs; sputter coated with gold and observed using an S-360 scanning electron microscope (Leo; Cambridge, USA).

Synthesis and characterization of colloidal silver nanoparticles

Silver nanoparticles (SN) were synthesized in accordance with a previously described procedure [18]. Briefly, in a tri-necked flask, 5 × 10⁻³ mol/l of silver nitrate (AgNO₃) (Merck KGaA, Germany) was reduced using 0.3 mol/l of sodium citrate (Na₃C₆H₅O₇) (Merck KGaA, Germany), followed by stabilization with a 1.4 mol/l solution of ammonia (NH₃) (Merck KGaA, Germany) at boiling

temperature. Once complete with the reduction of Ag⁺ ions to Ag⁰ (determined by the generation of an amber color), the colloidal SN were characterized by UV/Visible spectroscopy (Spectrophotometer Shimadzu MultSpec-1501, Shimadzu Corporation, Japan) and by X-Ray Diffraction (XRD) (Diffractometer Rigaku DMax-2000PC, Rigaku Corporation, Japan). The typical peak of SN centered at approximately 430 nm was observed in the UV/Vis spectrum, which established the colloidal stability and narrow particle size distribution [19].

*Application of silver nanoparticles and nystatin to pre-formed *Candida* biofilms*

After 48 h of single and dual species biofilm formation, AS was removed and the acrylic squares were washed once with 1 ml of PBS (0.1 M, pH 7). Then, 1 ml of 100 µg/ml of SN or nystatin (Bristol-Myers Squibb, UK) diluted in RPMI 1640 (Sigma-Aldrich, USA) was added to the pre-formed single and mixed biofilms and incubated at 37°C under agitation (120 rev/min) for an additional 24 h. For controls, single and dual species biofilms were pre-formed on acrylic squares and then incubated in RPMI without addition of SN or nystatin. The effect of SN or nystatin on pre-formed biofilms was examined by CV staining and by CFUs determination as described previously. All assays were performed in triplicate and 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 compared using a one-way ANOVA and a Bonferroni test. All tests were performed at a confidence level of 95%.

Results

Candida glabrata and C. albicans single and mixed species acrylic colonization

Both *C. glabrata* and *C. albicans* were able to adhere to the acrylic surface in the presence of AS after 2 h incubation (data not shown). Moreover, it was apparent that *C. glabrata* and *C. albicans* adhered similarly, without statistical differences between the species, even when used in dual species experiments ($P > 0.05$).

Two different approaches were employed to assess biofilm formation, enumeration of CFUs (Fig. 1A) and measurement of total biofilm biomass (Fig. 1B). It was evident that both species formed biofilms on acrylic after 48 h. It was interesting to note that the number of *C. glabrata* cells present in the biofilms was higher than those of *C. albicans*

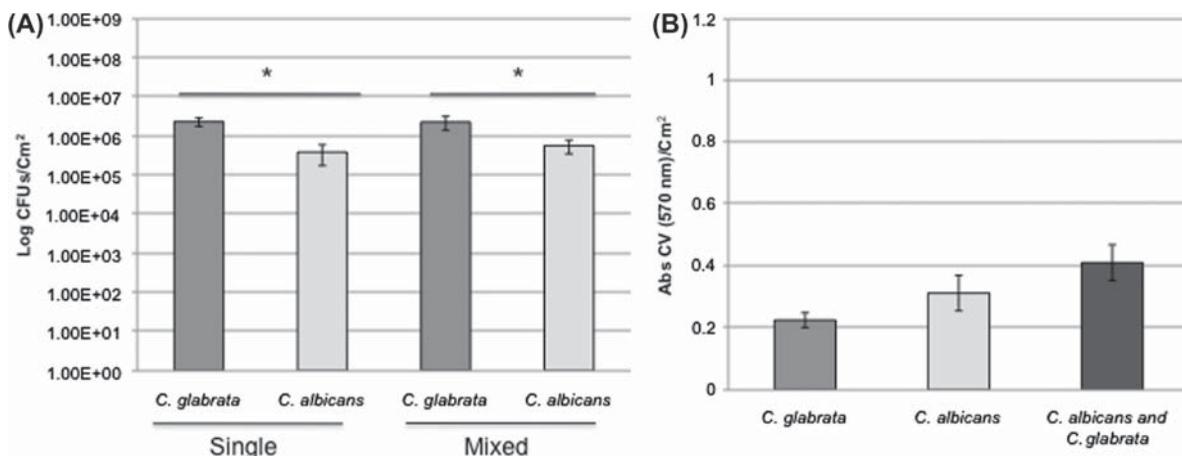


Fig. 1 Biofilm formation of *Candida glabrata* and *Candida albicans* after single and mixed colonization of acrylic resin. (A) Log₁₀ number of cells per unit area of acrylic; (B) Absorbance values of crystal violet (Abs CV) per unit area of acrylic. Error bars represent standard deviation. *Statistically significant ($P < 0.05$) differences.

cells in single and dual species experiments (Fig. 1A; $P < 0.05$). Moreover, the studies showed that there were no significant differences between single and dual species experiments in terms of the number of cultivable cells for a given species ($P > 0.05$). Statistical analysis revealed that *C. glabrata* and *C. albicans* biofilms were also similar in terms of total biomass ($P > 0.05$) (Fig. 1B).

SEM analysis of *Candida* adherence and biofilms on acrylic

SEM was used to analyze the adherence and biofilm structure for single and dual *Candida* species biofilms (Fig. 2). After 2 h incubation, *C. albicans* and *C. glabrata* adhered cells presented discontinuous distributions on the acrylic squares. Furthermore, *C. albicans* biofilms contained high quantities of hyphae and pseudohyphae. Interestingly, in dual species studies, SEM revealed an association between *C. glabrata* yeasts and the filamentous forms of *C. albicans*.

Biofilms of *C. albicans* consisted of cells with a variety of morphologies (hyphae, pseudohyphae and yeasts). The biofilms had a dense compact structure in AS, forming a multilayer that covered the entire acrylic surface. The biofilm structure of *C. glabrata* was less compact, with a thick layer of aggregated cells partially covering the acrylic surface.

SEM images of dual species biofilms revealed a multi-layer and dense biofilm with filamentous forms of *C. albicans* associated with small cells of *C. glabrata*.

Effect of silver nanoparticles and nystatin on pre-formed biofilms

To assess the effect of SN and nystatin on single and mixed *Candida* biofilms, 48 h pre-formed biofilms were treated

with 100 µg/ml of SN or 100 µg/ml of nystatin for 24 h. The effect of SN and nystatin against pre-formed biofilms was assessed based on the resulting number of CFUs (Fig. 3A) and total biofilm biomass (Fig. 3B).

Results (Fig. 3) showed that SN and nystatin were inhibitory to both *C. glabrata* and *C. albicans* biofilms generated on acrylic surfaces. To highlight this, there was a 10-fold reduction in *C. albicans* CFUs in single species biofilms treated with SN as compared with controls. An even greater (100-fold) reduction was evident when these biofilms were treated with nystatin. In the case of single species biofilms of *C. glabrata*, a 100-fold reduction in cell number occurred following treatment by either agent ($P < 0.05$). In dual species studies, reduction in the number of cells was again evident compared with respective controls ($P < 0.05$). The effect of nystatin on CFUs was again greater than that of SN (Fig. 3A). Results showed that SN significantly lowered *C. albicans* and *C. glabrata* biofilm biomass by approximately 2.5 and 2.4-fold, respectively ($P < 0.05$) (Fig. 4B). The effect of nystatin in reducing biomass was similar to SN for *C. albicans* ($P > 0.05$), but it was 2.3-fold higher again for *C. glabrata* ($P < 0.05$). Moreover, for mixed biofilms the effect of nystatin and SN was similar, with a reduction of approximately 1.5-fold ($P < 0.05$).

Discussion

While *C. albicans* is regarded as the principal agent of oral candidosis [1], other *Candida* species, such as *C. glabrata*, are increasingly involved, either as the sole agent [5,10] or through co-infection with *C. albicans* [5,6,10].

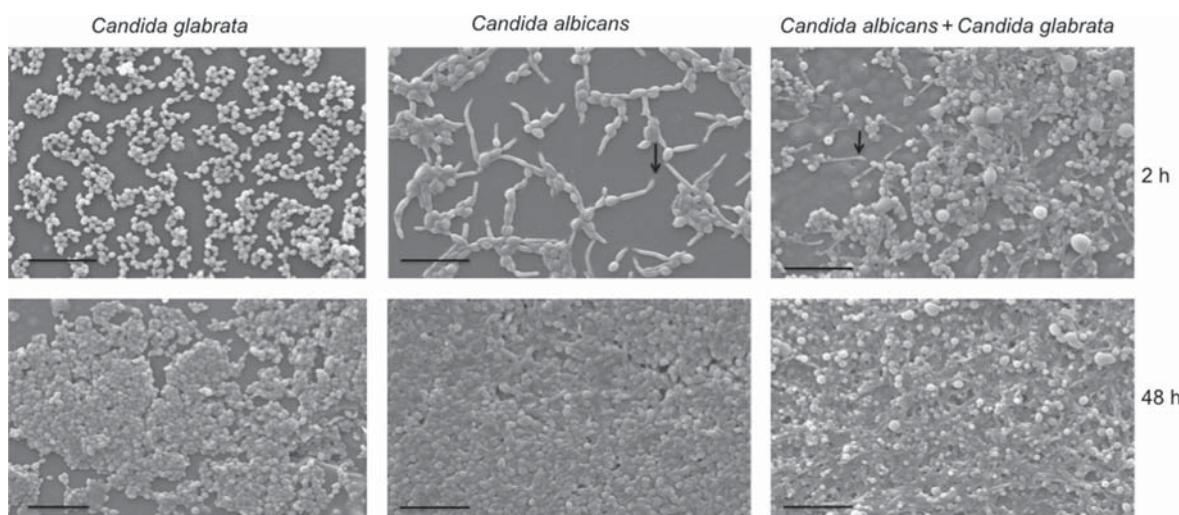


Fig. 2 Scanning electron microscopy of single and mixed colonization of acrylic by *Candida glabrata* and *Candida albicans* in artificial saliva at 2 h and 48 h incubation. Magnification $\times 1000$. Arrows indicate *Candida albicans* filamentous forms.

The initial objective of this work was to compare acrylic colonization and biofilm formation by *C. albicans* and *C. glabrata*, either by themselves or in association with each other (i.e., single and dual species studies). Results showed that both species adhered to a similar extent to acrylic resin with no apparent antagonistic effects when the two were combined on the resin surface (Fig. 2). These findings are in agreement with those in previous studies examining *Candida* species adhesion on the same medical device surfaces [13].

It is known that initial attachment of cells to a substratum is closely followed by cell division, proliferation, and biofilm development [20]. The current study demonstrated that under conditions that tried to mimic the oral environment by inclusion of artificial saliva, both species formed biofilms, although to different extents (Figs. 1A, 1B and 2). These results were in agreement with data from previous reports [21,22] concerning *Candida* species biofilm formation on a number of abiotic surfaces including acrylic. As noted earlier [17,23], *C. glabrata* biofilms yielded a lower total biomass compared with *C. albicans* (Fig. 1B) when CV staining was used as an indicator of biofilm formation. However, this direct comparison of biomass may be misleading as *C. glabrata* cells are physically smaller than *C. albicans* and *C. glabrata* are not able to generate larger filamentous forms as seen with *C. albicans* (Fig. 2). Interestingly, *C. glabrata* biofilms, despite having a lower biomass (Fig. 1B), contained higher numbers of cultivable cells compared with *C. albicans* biofilms (Fig. 1A).

The present study showed that even in dual species biofilms, the number of cultivable cells of each species was not affected by the presence of the other species (Fig. 1A). It is known that *C. glabrata* exhibits significant differences with *C. albicans*, including relative cell size (as mentioned

previously), morphology and biochemistry. *Candida glabrata* cells are smaller (1–4 μm) than *C. albicans* cells (4–6 μm) and have a narrower spectrum of carbohydrate utilization [3]. One possible explanation therefore for the relatively frequent occurrence of co-infection of *C. albicans* and *C. glabrata* is that these differences limit the extent of inter-species competition allowing the organisms to occupy similar oral niches, which could have important implications with respect to pathogenic potential. Interestingly, SEM images (Fig. 2) of mixed colonization studies revealed an association between *C. albicans* and *C. glabrata* cells which might conceivably aid colonization of the latter *in vivo* where there is exposure to removal by salivary flow. In this context, Ez-Azizi *et al.* [24] showed the efficient adherence of *C. albicans* to pre-formed *C. glabrata* biofilms in a catheter model and also suggested the possible co-aggregation of these two species.

A biofilm environment is an important characteristic in promoting persistence of *Candida* in the host, as biofilms have a greater resistance to removal by host factors and are more tolerant to administered antifungal therapies [25]. *Candida* cells are indeed adept at forming biofilms on acrylic, as evident in this study (Figs. 1–3). It is this property of biofilms that has led to the recognition of an urgent need to find new and alternative antimicrobial approaches that are effective against biofilms.

Nystatin is a polyene antifungal that is frequently used as a topical agent in the treatment of oral candidosis [1]. The bactericidal effects of silver-based treatments such silver nanocomposites and SN has recently also been established [26]. SN has also been reported to exhibit inhibitory effects on bacterial biofilm formation [27,28], as well as candidal biofilm formation [15]. However, the antimicrobial

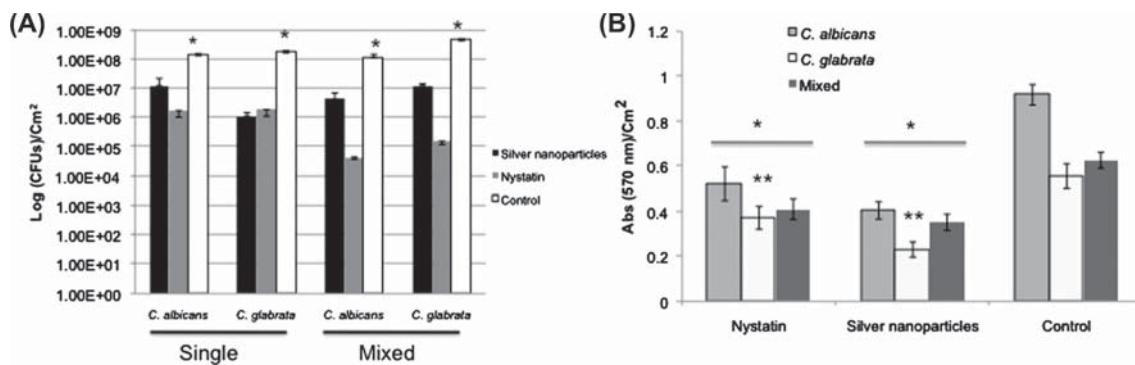


Fig. 3 Effect of 100 µg/ml of silver nanoparticles or 100 µg/ml of nystatin on single and mixed pre-formed biofilms of *Candida glabrata* and *Candida albicans*. The biofilms were analyzed after 24 h of treatment by determination of the number of cultivable cells (A), or quantification of total biomass (B). *Results statistically different from the respective control ($P < 0.05$); ^aStatistically significant ($P < 0.05$) single and mixed colonization results; **Significantly significant ($P < 0.05$) effects of nystatin and silver nanoparticles.

activity of SN against pre-formed *Candida* biofilms has received comparatively little attention. Consequently, the present study evaluated the antimicrobial effects of nystatin and SN on pre-formed single and dual species (*C. albicans* and *C. glabrata*) biofilms on acrylic. Previous investigations have identified the fungicidal effects of these two agents relative to the MICs of nystatin (1–2 µg/ml) and SN (0.4–3.3 µg/ml) against both species [15]. In the present study, experiments on pre-formed biofilms revealed that both SN and nystatin significantly inhibited *C. glabrata* and *C. albicans* biofilms in terms of the number of cultivable cells (Fig. 3A) and total biomass (Fig. 3B). With regards to the number of CFUs recovered from single-species biofilms, it was evident that SN had a similar effect as nystatin against *C. glabrata*, but was less effective against *C. albicans* (Fig. 3A). Interestingly, the effect of nystatin was greater on mixed species biofilms as compared with SN. Compared with single species biofilms, the effect of nystatin was lower against dual species biofilms, a feature not apparent with SN treatment (Fig. 3A).

In the present work, CV staining was used to quantify total biofilm biomass since nystatin and SN may also have had effects on the biofilm matrix. Results showed (Fig. 3B) that both agents caused a significant reduction in total biomass, with SN being more active against single *C. glabrata* biofilms than nystatin. Indeed, it is known that significant differences can occur in the chemical composition and structure of the matrix of single and mixed species biofilms [11]. It is important to note that the total biomass of mixed species biofilms was similar or lower than that due to single species (Fig. 3B). This could be indicative of a lower quantity of biofilm matrix in the dual species biofilms, which could in turn facilitate greater nystatin penetration into it. The extracellular polysaccharide of the biofilm is a recognized inhibitor/barrier to diffusion of antimicrobial agents with an ability to ionically bind to certain agents as

they diffuse through the biofilm, thereby effectively reducing ‘bio-availability’ [1]. In the case of SN, it has been reported that interactions of the nanoparticles with micro-organisms in biofilms induces particle aggregation [29]. Stewart and Franklin [30] reported that biofilm features such as oxygen concentration, the biofilm substrate, pH and biofilm composition may influence this SN aggregation and consequently the diffusion and effect on biofilm cells. These findings may have significance concerning the similar activity of SN against single and mixed biofilms in these studies. The concentration and size of SN showed to be effective in this work was lower than that previously reported by others authors to be toxic *in vitro* against human cells [31,32].

In summary, this work showed that *C. glabrata* and *C. albicans* oral isolates were able to colonize acrylic surfaces in the presence of AS and to co-exist in dual species biofilms without antagonism. This observation may have been anticipated as it reflects the frequently recorded incidences of oral candidosis associated with the presence of these two *Candida* species. Additionally, it was shown that both nystatin and SN exhibited antifungal activity against both single and dual species pre-formed biofilms. Although nystatin had a greater effect in reducing the number of viable cells in the biofilms compared with SN, the reduced solubility of nystatin *in vivo* remains problematic. Thus, given the inhibitory effects of SN against candidal biofilms seen in the present study, its use as an alternative treatment for oral candidosis (particularly those involving acrylic biofilms) should be considered.

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