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Synergistic activity of lysozyme and antifungal agents against *Candida albicans* biofilms on denture acrylic surfaces

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ABSTRACT

Denture related oral candidiasis is a recalcitrant fungal infection not easily resolved by topical antifungals. The antimycotic protein lysozyme, in saliva is an important host defense mechanism although its activity against *Candida* biofilms on denture acrylic has not been evaluated.

Objectives: (i) To establish a clinically relevant denture acrylic assay model to develop standardized *Candida albicans* biofilms, and (ii) assess the inhibitory effects of lysozyme alone and, the latter combined with antifungals (nystatin, amphotericin B, ketoconazole and 5-fluorocytosine) on sessile *Candida* cells and, finally (iii) to visualize the accompanying ultrastructural changes.

Design: The rotating-disc biofilm reactor was used to develop standardized 48 h *Candida* biofilms on acrylic discs in YNB/100 mM glucose medium and the biofilm metabolic activity was monitored using a tetrazolium reduction assay.

Results: The biofilm metabolic activity was similar in 18 identical denture acrylic discs ($p < 0.05$) thus validating the rotating-disc biofilm model. Very low concentrations of lysozyme (6.25 μg/ml) significantly ($p < 0.01$) inhibited *Candida* biofilm formation indicating that lysozyme may likely regulate intra-oral *Candida* biofilm development. Although 100 μg/ml lysozyme killed 45% of sessile *Candida* cells, further increasing its concentration (up to 240 μg/ml) had no such effect. Nystatin, amphotericin B, and ketoconazole in association with 100 μg/ml lysozyme exhibited effective synergistic killing of biofilm *Candida* in comparison to drug-free controls. Scanning electron and confocal scanning laser microscopy analysis confirmed the latter trends.

Conclusion: Our results indicate that agents found in biological fluids such as lysozyme could be a safe adjunct to antifungals in future treatment strategies for recalcitrant candidal infections.

1. Introduction

Candida-associated denture stomatitis is a recalcitrant disease in some 60% of otherwise healthy denture wearers.[1] It usually manifests as an erythematous and an edematous area of the denture bearing mucosa[2,3] and, a definitive association between *Candida* species, particularly *Candida albicans* and the pathogenesis of the disease has been established.[4–7] However, the etiology of the disease is multifactorial and other factors associated with its pathogenesis include denture

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trauma, a high carbohydrate diet, Sjörgrens syndrome, low salivary flow leading to xerostomia.[8–10] Under the latter circumstances lack of salivary antifungal defences such as lysozyme is thought to aggravate *Candida* infection.

Lysozyme, a component of the host non-specific immune system found in saliva[11] has significant antifungal activity against the planktonic form of several *Candida* species.[12,13] Nevertheless, there is no specific information on the antifungal effect of salivary lysozyme in regulating biofilm mode of *Candida* growth in the oral cavity. In clinical terms, a mouthwash containing lysozyme and lactoferrin accompanied with itraconazole therapy was shown to be an effective alternative to antifungal treatment alone in the management of severe refractory oral candidiasis.[14] A recent study has also demonstrated that lysozyme combined with the imidazole lanoconazole *in vitro* has synergistic antifungal activity against *C. albicans* blastospores.[15] Despite the synergism shown between antifungals and lysozyme, no studies to our knowledge, have been conducted to investigate the efficacy of lysozyme either with or without antifungals in eradicating biofilm or sessile *Candida*, on denture acrylic surfaces, as opposed to their planktonic or suspended phase counterparts.

Therefore, the aims of the present study were to (i) assess the dose and time dependent inhibitory effects of lysozyme on *C. albicans* biofilms, (ii) asses the synergistic effect, if any, of lysozyme and four commonly used antifungals, i.e., amphotericin B, nystatin, ketoconazole and 5-fluorocytosine on the susceptibility of *C. albicans* biofilms and, (iii) investigate the associated ultrastructural changes of *Candida* biofilms using scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM).

2. Materials and methods

2.1. *Candida strains and growth conditions*

Two *Candida* strains, *C. albicans* ATCC 90028 from the American Type Culture Collection and a clinical oral isolate, *C. albicans* (192887 g) from a human immunodeficiency virus-infected individual from Hong Kong were used in this study. The strains were stored in vials with multiple glass beads (Microbank, Pro-Lab Diagnostics) at −70 °C, subcultured monthly on Sabouraud's dextrose agar (SDA, Oxoid Ltd., Hampshire, UK) and maintained at 4 °C during the experimental period. Purity of the phenotypes were further confirmed by API32 assimilation tests (BioMérieux, Marcyl'Etoile, France), germ tube test, and CHROMagar™ Candida (CHROMagar, Paris, France) technique.[16]

2.2. *Preparation of the standard Candida cell suspension*

The test *C. albicans* strain was cultured on SDA (SDA, Oxoid Ltd., Hampshire, UK), at 37 °C for 18 h. To prepare the yeast inoculum for biofilm growth, a loopful of the candidal growth was transferred into 100 ml of liquid yeast nitrogen base (YNB; Difco) supplemented with 50 mM glucose and incubated for 18 h at 37 °C in a rotary shaker (at 75 rpm). Cells were harvested and washed twice with 25 ml phosphate-buffered saline (PBS; pH 7.2) by centrifugation (4000 × *g*; 5 min) and resuspended in YNB supplemented with 100 mM glucose to a concentration of 10^7 CFUs/ml by adjusting the optical density to MacFarland 4 opacity.

2.3. *Preparation of denture acrylic discs*

Polymethylmethacrylate heat-cured acrylic (pro-Base hot-Ivoclor) was used to prepare rods (8 mm diameter × 75 mm in length) embedded in plaster of Paris. The rods were then sliced using a lathe to prepare acrylic discs (5 mm diameter × 1.5 mm thick). In order to simulate the palatal surface of an acrylic denture the surface of these discs were polished in a standard manner using grit p600c waterproof silicon carbide paper (English Abrasives Limited, UK.) to resemble the palatal surface of the denture appliance.

Acrylic discs were disinfected by immersing in a 0.5% sodium hypochlorite solution for 3 min. The sterility of the discs was checked by rolling the discs on SDA plates and incubating at 37 °C for 24 h, and by incubating in PBS at 37 °C overnight and observing for yeast growth by Gram staining.

2.4. *Hen egg white lysozyme*

Hen egg white lysozyme (Lz) (Sigma Chemical Co., Poole, UK) was used in all experiments. A stock solution of Lz (3000 μg) was prepared with sterile distilled water, stored at 4 °C, and used within a week.

2.5. *Antifungal agents*

Five different antifungals were used in the experiments as described previously.[17] Amphotericin B (AmB; Sigma, USA) was dissolved in dimethylsulphoxide and absolute ethanol (3:2 ratio); nystatin (Nys; Sigma, USA) and the azole ketoconazole (Keto; Sigma, USA) in dimethylsulphoxide and 5-fluorocytosine (5-FC; Sigma, USA) in sterile water. The MIC of each of the antifungals was determined by the standard NCCLS broth microdilution method.[18]

2.6. *Biofilm development and growth quantification*

Candida biofilms were developed on acrylic discs using the methodology described by Hentzer et al.[19] The system consists of a reactor vessel and a magnetic stir disk (for continuous mixing) carrying 18 sample ports to insert substrate discs on which biofilms can be developed.

C. albicans ATCC 90028 was used to develop standard biofilms on acrylic discs asceptically placed into each of the 18 sample ports of the magnetic stir disk. The reactor vessel was then filled with 100 ml of YNB/100 mM glucose containing the standard yeast cell suspension (10^7 CFUs/ml) and was initially incubated for 90 min at 37 °C in an orbital shaker incubator at 75 rpm to develop the adhesion phase of the *Candida* biofilm. Following the adhesion phase, the stir disk was gently rinsed in 100 ml phosphate-buffered saline (PBS; pH 7.2), aseptically transferred to a second sterile reactor vessel which has a stage fixed with a magnetic stirrer and 200 ml of the respective nutrient medium (YNB/100 mM glucose). This vessel was

placed in the orbital incubator (37 °C; 50 rpm) and was connected to a nutrient bottle, waste bottle and an infusion pump to complete the *in vitro* experimental system. After the desired flow rate of the nutrient medium was established in the model (chemostat mode at a dilution rate of 0.10 h^{-1}) biofilm formation was allowed to proceed for 48 h. Finally, the rotating wheel with the 18 acrylic discs was asceptically removed and immersed in 100 ml sterile PBS to remove non-adherent cells and the discs gently removed for further investigations.

For all other investigations *C. albicans* (192887 g), a clinical isolate was used to develop 48 h biofilms. The biofilms on the acrylic discs were quantified using XTT ([2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]) reduction assay as described previously.[20] In brief, XTT (Sigma, St. Louis, MO) solution (1 mg/ml in PBS) was prepared, filter sterilized using a 0.22-μm-pore-size filter, and stored at −70 °C. Menadione (Sigma) solution (0.4 mM) was also prepared and filter sterilized immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with the menadione solution at a ratio of 5–1 by volume.

2.7. The kinetics of C. albicans biofilm growth in lysozyme incorporated nutrient medium

To study the effect of a nutrient medium with varying concentrations of lysozyme on biofilm viability we used, another assay system where acrylic discs were placed in commercially available pre-sterilized, polystyrene, flat-bottom 96-well microtitre plates (Iwaki, Tokyo, Japan).[20] For the adhesion phase, sterile acrylic discs were placed in the wells of a microtitre plate containing 0.75 ml of the standard *Candida* inoculum in YNB/100 mM glucose medium with the following concentrations of lysozyme; 6.25, 12.5, 25, 50 and 100 μg/ml. A negative control without lysozyme was also included. The microtitre plate with discs was incubated in a orbital incubator (75 rpm) at 37 °C for 90 min. At the end of the adhesion phase the acrylic discs were gently removed, rinsed in PBS and placed in new microtitre plate wells containing fresh nutrient medium with the varying lysozyme concentrations (6.25, 12.5, 25, 50 and 100 μg/ml) and incubated in a orbital incubator (75 rpm) at 37 °C for total period of 24 h. At four different time points (0, 8, 12 and 24 h) during the post-adhesion period four acrylic discs were removed gently, rinsed in PBS and the biofilm development on each disc was quantified by the XTT reduction assay. This experiment was performed in sets of four replicates on two separate occasions.

2.8. The sensitivity of C. albicans biofilms to a range of lysozyme concentrations

The 48 h *Candida* biofilms on denture acrylic discs were developed in the rotary disc reactor as described earlier. Each disc with the intact biofilm was placed in 2 ml plastic eppendorf tubes containing 1 ml of a series of lysozyme concentrations (60, 120, 180, 240 μg/ml) and incubated for 24 h at 37 °C. A negative control was also included. The cells were then harvested by centrifugation (13,200 × *g* for 10 min). The supernatant was discarded and then 200 μl of PBS and 12 μl of the XTT-menadione solution were added to each of the eppendorf tubes. The tubes were then incubated in the dark for 3 h at 37 °C. Following centrifugation at 13,200 × *g*/10 min, 100 μl of the supernatant was transferred into wells of a microtitre plate and the color change in the solution was measured with a microtiter plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices Ltd., Sunnyvale, CA) at 492 nm.

2.9. Comparison of in vitro susceptibility of mature C. albicans biofilms to lysozyme and antifungal combinations

To determine the fungicidal activity of simultaneous incubation with 100 μg/ml lysozyme and a series of increasing concentrations of antifungals, i.e., amphotericin B, nystatin, ketoconazole and, 5-fluorocytosine the following test conditions were used. Two day *Candida* biofilms on acrylic discs were obtained as described above and gently rinsed in PBS and incubated individually with a combination of 100 μg/ml lysozyme (which was shown to have an approximate 45% reduction in metabolic activity when compared with the control in earlier experiments) and a series of antifungal dilutions as follows. Thus the polyenes (amphotericin B, nystatin) and 5-fluorocytosine were added to 4.5 ml of 100 μg/ml lysozyme to yield final concentrations of ×8, ×12, ×16 and ×24 MICs of antifungals whereas the final concentrations of the azole were ×16, ×24, ×48 and ×96 MIC.

Each of these combinations of antimycotics were incubated with 48 h biofilms at 37 °C in a rotary incubator (50 rpm) for a period 24 h. Two negative controls were used; a lysozyme and antifungal-free suspension and the biofilm incubated with 100 μg/ml of lysozyme alone. The metabolic activity of the biofilms were subsequently measured by the XTT reduction assay as described previously. Each of the antifungals was tested on three separate occasions with three determinations on each occasion.

2.10. Scanning electron microscopy

For SEM, the intact 48 h *Candida* biofilms were rinsed gently with PBS and post-fixed in v/v 0.1 M phosphate buffer at 4 °C overnight and vapor fixed in 1% osmium tetroxide for 2 h at room temperature.[21] Samples were subsequently washed in distilled water, dehydrated gently by washing in a series of ethanol alcohol (70% for 1 h, 95% for 10 min and 100% for 10 min), and air dried in a desiccator prior to sputter coating with gold. Afterwards, specimens were mounted on the aluminium stubs and coated with gold in a low-pressure atmosphere with an ion sputter coater (Jeol JFC1 100: Jeol, Tokyo, Japan). The topographic features of the biofilm were visualized with a scanning electron microscope (Philips XL30CP) in high vacuum mode at 10 kV and the images were processed for display with Photoshop software (Adobe Systems Inc., Mountain View, CA).

2.11. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) studies were carried out to visualize and quantify the inhibitory effect of

different concentrations of lysozyme, antifungals (nystatin, amphotericin B, ketoconazole and 5-fluorocytosine) and, a combination of the latter with lysozyme on 48 h *Candida* biofilms developed on denture acrylic discs. Controls without the antimicrobials were also included. The 48 h biofilms were gently transferred into a 12-well microtitre plate and rinsed with PBS for 15 s. The discs were then immersed in 1 ml of the antimicrobial agents and incubated at 37 °C in an aerobic incubator for 24 h. Subsequently, the antimicrobial was removed and the viability of the biofilm cells was assessed by Molecular Probes LIVE/DEAD BacLight Bacterial viability kit which comprise SYTO-9 and proidium iodide (PI) (Molecular Probes, Eugene, OR). After incubation with the dyes, the polymethylmethacrylate discs with biofilms were placed on glass slides and live/dead ratio of biofilm cells quantified using the CSLM system (Fluoview FV 1000, Olympus, Tokyo, Japan).[22]

2.12. Data analysis

T-Test was used to determine significant differences in the metabolic activity of 18 biofilms developed on denture acrylic discs in the rotary incubator on two different occasions and, One-way ANOVA was used to determine (i) the effect of varying concentrations of lysozyme on biofilm growth during a 24 h period and, (ii) significant differences in the metabolic activity between untreated biofilms and biofilms treated with lysozyme and varying concentrations of the tested antifungals.

3. Results

3.1. Standardization assay

No significant differences were observed in the metabolic activity of 48 h *Candida* biofilms that developed on 18 acrylic discs, on two separate occasions ($p = 0.351$) when analysed using repeated measures analysis of variance. The mean colorimetric readings between 18 replicate biofilms within each experiment ranged from 0.690 ± 0.05 to 0.706 ± 0.04 and the coefficient of variance for the two experiments was 7.0 (unpublished data). The scanning electron microcopic visualization of the 48 h *Candida* biofilm on a denture acrylic disc is shown in Fig. 1(a) and (b). The morphology of the 48 h *Candida* biofilm was striking, and comprised of multilayers of abundant blastospores and profuse hyphal elements intricately woven together within a structured matrix of extracellular material. The biofilm architecture was further characterized by abundant water channels.

3.2. The kinetics of C. albicans biofilm growth in lysozyme incorporated nutrient medium (YNB/100 mM glucose)

The kinetics of *Candida* biofilm formation over a 12 h period on denture acrylic discs in the presence of varying concentrations of lysozyme, as determined by the colorimetric, XTT-reduction assay are illustrated in Fig. 2. Statistical analysis revealed that the metabolic activity, was significantly ($p < 0.01$) high in

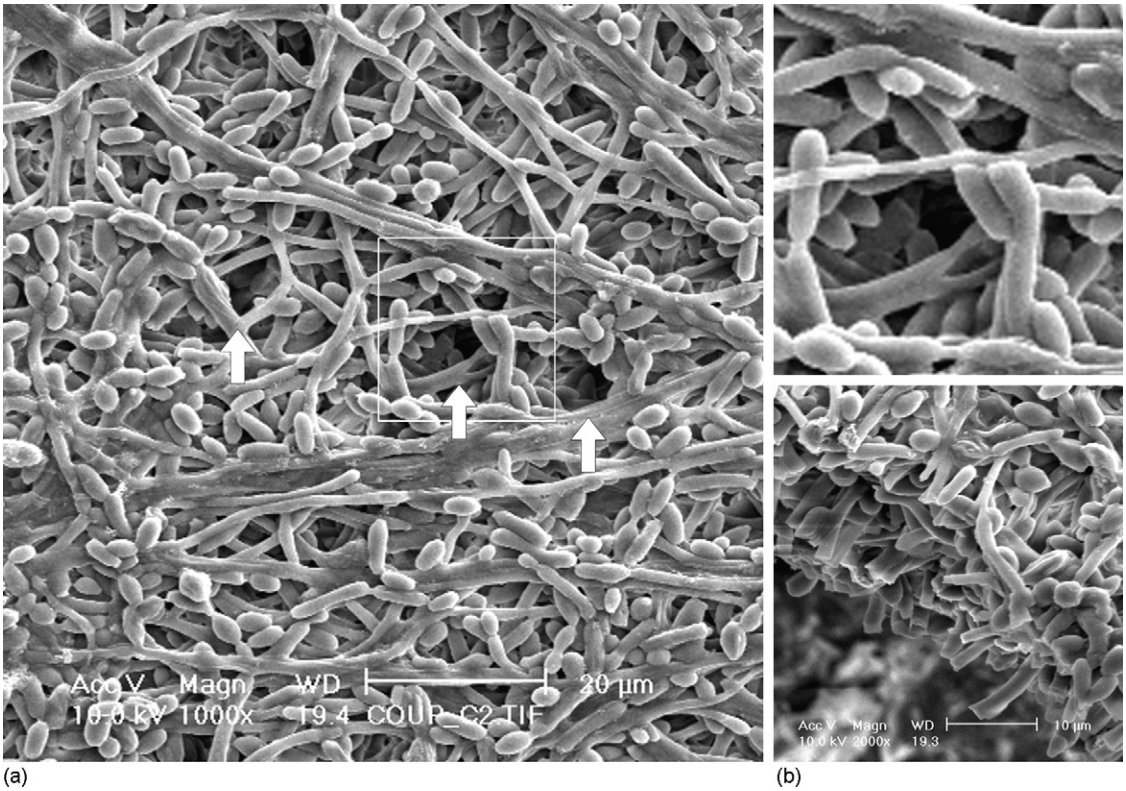

Fig. 1 – (a) Scanning electron microscopic visualization of a 48 h *C. albicans* biofilm grown on a denture acrylic disc showing the compact nature of yeast and filamentous forms deeply embedded within a matrix of dense extracellular material (note the large number of water channels (arrows) within the complex biofilm matrix), (b) enlarged image of the multilayered biofilm ultrastructure comprising intricately woven hyphal elements and attached blastospores that are less predominant.

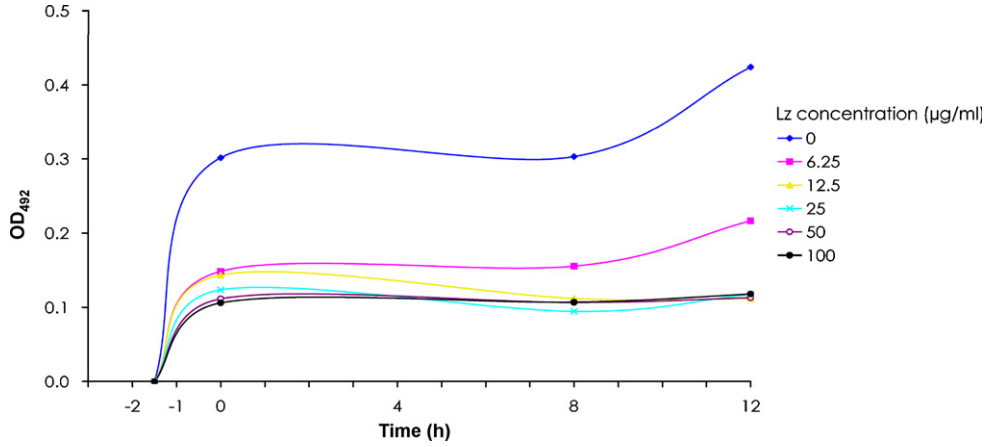

Fig. 2 – Biofilm development of *C. albicans* during a 12 h period after exposure to a nutrient medium (YNB/100 mM glucose) incorporated with a range of lysozyme concentrations. Each symbol represents the mean of eight determinations with quadruplicate samples on two different occasions. The metabolic activity of biofilms in lysozyme-free medium was significantly higher ($p < 0.05$) compared with those developed in lysozyme incorporated media.

biofilms formed in lysozyme-free medium (control) compared with those containing increasing concentrations of lysozyme (6.25–100 μg/ml) ($p < 0.05$).

In chronological terms, after 8 h of incubation there were significant differences ($p < 0.05$) in the metabolic activity between biofilms in media with 6.25 and 12.50 μg/ml lysozyme and, the control biofilms. However, further increases in lysozyme concentrations (up to 100 μg/ml) or the incubation time (up to 12 h) did not result in further significant changes in the metabolic activity. In general, during the 24 h period of incubation the metabolic activity of lysozyme-laced wells decreased 59% (0.356–0.146).

3.3. *The sensitivity of 48 h C. albicans biofilms to a range of lysozyme concentrations*

When 48 h *Candida* biofilms were incubated for a period of 24 h in a nutrient medium with lysozyme concentrations of 60, 120, 180 and 240 μg/ml, the percentage reduction in the metabolic activity of the biofilms compared to the lysozyme-free control were 28.19%, 52.71%, 64.43% and 69.62%, respectively (Fig. 3(A)). This trend in the biofilm destruction was also evident in scanning electron micrographs where biofilm cell degradation was increasingly pronounced in direct proportion to lysozyme concentrations (Fig. 3(B)). An observation further confirmed by CSLM data wherein 97.15%, 74.01%, 63.53%, 44.56% and 15.39% viability were noted for controls, 60, 120, 180 and 240 μg/ml of lysozyme suspensions, respectively (Fig. 3(C)).

3.4. *Susceptibility of 48 h C. albicans biofilms to varying concentrations of four antifungal agents (i.e., amphotericin B, nystatin, ketoconazole and 5-fluorocytosine) and 100 μg/ml lysozyme*

A screening experiment to ascertain the optimal drug concentration to study synergism of anifungals and lysozyme yielded the following minimum inhibitory concentrations for amphotericin B, nystatin, ketoconazole and 5-fluorocytosine; 0.1, 0.78, 32 and 0.098 μg/ml, respectively.

3.5. *Amphotericin B*

Susceptibility of 48 h *Candida* biofilms to 100 μg/ml lysozyme compared with the lysozyme free control, demonstrated a highly significant 49.05% reduction (0.66–0.34; $p < 0.001$) in the metabolic activity of the biofilm as measured by the XTT assay, after a 24 h incubation period (Fig. 4, Table 1).

A significant percentage reduction in metabolic activity was noted in *Candida* biofilms treated with 100 μg/ml lysozyme laced with ×12 MIC (70.12%; 0.34–0.19; $p < 0.001$), ×18 MIC (72.88%; 0.34–0.18; $p < 0.001$) and, ×24 MIC (80.19%; 0.34–0.13; $p < 0.001$) of amphotericin B when compared with the untreated control (lysozyme and drug-free) (Table 1). Overall, the increased concentrations of the drug was not particularly efficacious in killing sessile *Candida* cells compared to its lower concentration of ×12 MIC.

Nevertheless, when compared with the inhibitory effect of 100 μg/ml lysozyme alone the increased percentage reduction in cell viability when lysozyme (100 μg/ml) was supplemented with ×6, ×12, ×18 and ×24 MIC of amphotericin B were; 10.19%, 35.29%, 41.37% and 58.55%, respectively. This indicates a synergistic effect of 100 μg/ml lysozyme with increasing concentrations of the antifungal. The percentage survival of biofilm cells in comparison to both controls are shown in Table 1.

3.6. *Nystatin*

A combination ×6 MIC nystatin + lysozyme (100 μg/ml) and ×12 MIC nystatin + lysozyme (100 μg/ml) resulted in 66.71% (0.95–0.32; $p < 0.001$) and 79.30% (0.95–0.20; $p < 0.001$) reductions in metabolic activity, respectively, when compared with 100 μg/ml lysozyme controls indicating synergy. As expected a dose dependent significant difference ($p < 0.01$) in the killing effect was also seen between ×6 MIC and ×12 MIC concentrations of nystatin (and 100 μg/ml lysozyme) (Table 1). The percentage survival of biofilm cells treated with varying concentrations of nystatin and 100 μg/ml lysozyme is shown in Table 1.

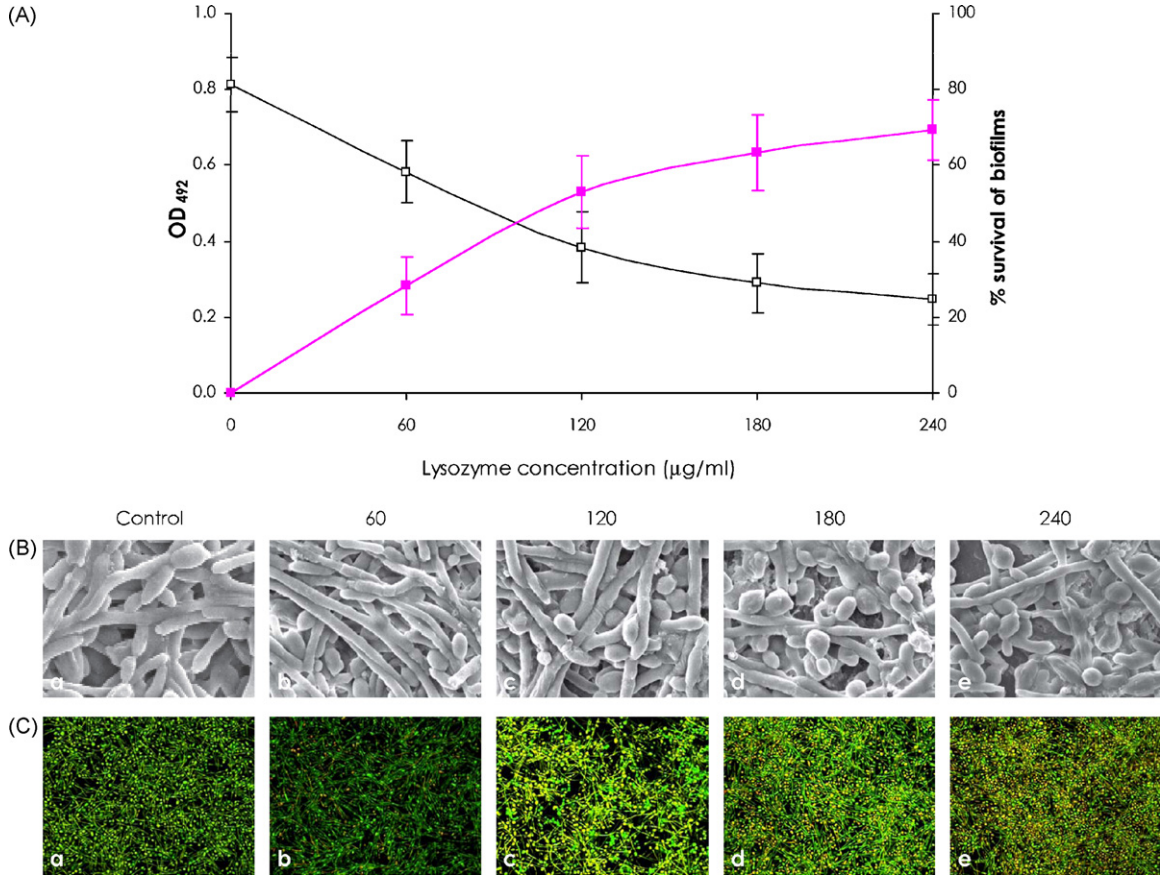

Fig. 3 – (A) The sensitivity of 48 h *C. albicans* biofilms to a range of lysozyme concentrations as determined by the colorimetric XTT-reduction assay (left axis) and confocal scanning laser microscopy (right axis). (B) Scanning electron microscopic images of 48 h *C. albicans* biofilms on denture acrylic discs treated with lysozyme; (a) untreated control biofilm in YNB/100 mM glucose medium and biofilms treated with, (b) 60 μg/ml lysozyme, (c) 120 μg/ml lysozyme, (d) 180 μg/ml lysozyme and (e) 240 μg/ml lysozyme. (C) Confocal scanning laser microscopic images of 48 h *C. albicans* biofilms on denture acrylic discs treated with varying concentrations of lysozyme, highlighting the live (green) and dead (red) sessile cells stained with LIVE/DEAD BacLight Bacterial Viability Kit (a) untreated *Candida* biofilm cells and *Candida* biofilm cells treated with (b) 60 μg/ml lysozyme, (c) 120 μg/ml lysozyme, (d) 180 μg/ml lysozyme and (e) 240 μg/ml lysozyme. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Summarizing these results for the polyenes, we noted that the combined effect of 100 μg/ml lysozyme and a ×12 MIC concentration of either amphotericin B or nystatin showed the most significant fungicidal effect with survival rates of 29.9% and 20.9%, respectively and, further increasing the drug concentrations had no significant increased killing effect.

3.7. *Ketoconazole*

Candida biofilms treated with a combination of ×12 MIC ketoconazole and 100 μg/ml lysozyme showed a significant 67.61% synergistic inhibition of the metabolic activity (i.e., 0.69–0.22; $p < 0.001$) compared with the lysozyme and drug free control and a much less inhibition of 29.65% (0.34–0.22; $p < 0.05$) when compared with biofilms treated with 100 μg/ml lysozyme alone indicating synergism. Further increases in ketoconazole concentration up to ×48 MIC combined with 100 μg/ml lysozyme was not more effective in inhibiting biofilm metabolic activity. The percentage survival of biofilms treated with 100 μg/ml lysozyme and ketoconazole is shown in Table 1.

3.8. *5-Fluorocytosine*

As opposed to the polyenes and the azole a range of 5-fluorocytosine concentrations (×6 and ×24 MIC) was not significantly ($p > 0.05$) effective in inhibiting *Candida* biofilm metabolic activity, compared with biofilms treated with 100 μg/ml lysozyme alone (Table 1). This demonstrates that 5-fluorocytosine does not elicit a synergistic fungicidal effect in combination with 100 μg/ml lysozyme.

Taking into account the results of the foregoing experiments we noted that ×12 MIC concentrations of nystatin, amphotericin B and ketoconazole was the optimal drug concentration which kills most of biofilm cells. Hence another set of experiments was performed to evaluate simultaneously the effect of these agents in the 18 disc test system using all requisite control systems, namely; lysozyme and drug free

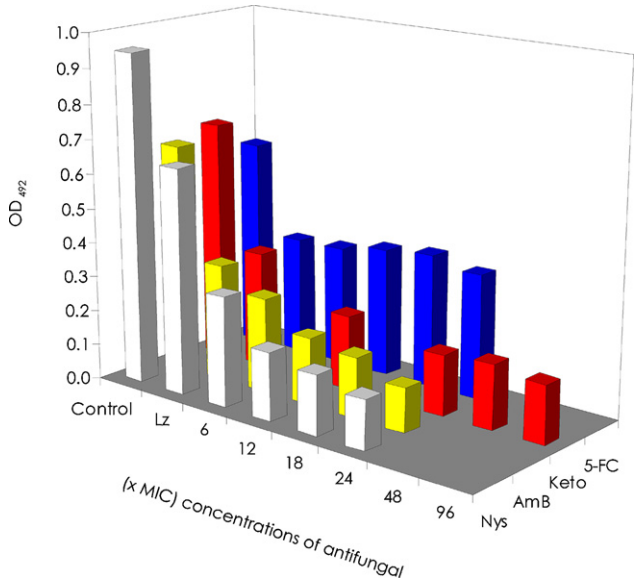

Fig. 4 – A composite graph showing the susceptibility of 48 h *C. albicans* biofilms on denture acrylic discs to varying concentrations of the four antifungal agents (i.e., nystatin, amphotericin B, ketoconazole and 5-fluorocytosine) in association with 100 μg/ml lysozyme (please see Table 1).

control, 100 μg/ml lysozyme and ×12 MIC concentrations of nystatin and 5-fluorocytosine. We noted that a concentration of 100 μg/ml lysozyme alone showed 36% reduction (0.85–0.55; $p < 0.001$) in 48 h *Candida* biofilm metabolic activity when compared with the lysozyme-free control biofilm. A ×12 MIC concentration of nystatin demonstrated a significant 70.9% inhibitory effect when compared with the lysozyme-free control biofilm (0.85–0.24; $p < 0.001$) and 52.19% when compared with 100 μg/ml lysozyme (0.55–0.24; $p < 0.001$). A concentration of ×12 MIC nystatin in association with 100 μg/ml lysozyme showed a synergistic killing effect of 81.42% when compared with both the lysozyme-free control

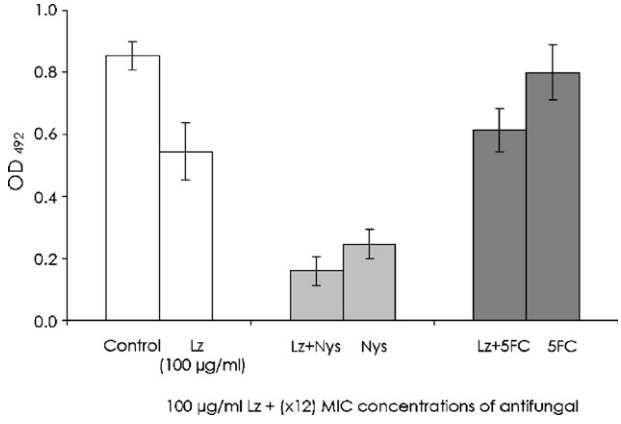

Fig. 5 – The inhibition of *C. albicans* biofilms on denture acrylic discs treated with 100 μg/ml lysozyme, nystatin, and 5-fluorocytosine plus the drugs in association with 100 μg/ml lysozyme. The reduction of biofilm metabolic activity was measured using the XTT reduction assay (please see Table 2).

($p < 0.001$) and 67.7% compared with 100 μg/ml lysozyme ($p < 0.05$) (Table 2, Fig. 5).

In contrast, ×12 MIC concentration of 5-fluorocytosine with 100 μg/ml lysozyme failed to demonstrate any synergistic inhibition of biofilm metabolic activity compared with either the lysozyme-free control (0.85–0.61) or 100 μg/ml lysozyme (0.61–0.55). The percentage survival of biofilm cells for the above drug combinations is shown in (Table 2, Fig. 5).

3.9. *Scanning electron microscopy*

Untreated cells in the control *Candida* biofilms showed characteristic oval blastospores with a smooth surface morphology entangled in a thick biofilm mass Fig. 6(A) (a). In (×12 MIC) amphotericin B, nystatin and ketoconazole treated biofilms, the cells were ruptured, damaged or bloated with most cell walls demonstrating a rough, irregular topograpic features (Fig. 6(A), b–e). However, the most cellular damage and disruption was seen in biofilms treated with both 100 μg/ml lysozyme and antifungals especially ×12 MIC nystatin and amphotericin B (Fig. 6(A), g–j). Ketoconazole treated sessile cells retained their smooth cell surface and elongated shape although arrested growth of blastospores and a few swollen, deformed and elongated hyphae were also visible. Finally, very little morphological changes were noted in biofilm *Candida* treated either with 5-fluorocytosine or the latter plus 100 μg/ml lysozyme (Fig. 6(A), e and j).

These ultrastructural changes in sessile cells treated with the four antifungals visualized by scanning electron microscopy were confirmed using confocal scanning laser microscopy assays. As shown in Fig. 6(B) (a) the untreated control biofilms were predominantly viable (green) with only a few red stained non-viable cells interspersed. Fig. 6(B) (b–e) shows the ultrastructural features of (×12 MIC) nystatin, amphotericin B, ketoconazole and 5-fluorocytosine treated 48 h *Candida* biofilm cells. On CSLM computation of viable/nonviable cells nystatin treated cells exhibited the lowest number of viable cells compared with amphotericin B, ketoconazole and 5-fluorocytosine. The percentage survival of biofilms treated with nystatin, amphotericin B, ketoconazole and 5-fluorocytosine were 46.7%, 69.7%, 89.7% and 99.3%, respectively, while the percentage survival of biofilms treated with the foregoing antifungals in combination with 100 μg/ml lysozyme were 16.7%, 49.8%, 60.3% and 60.9%, respectively.

4. Discussion

It is well recognized that the adherence of *Candida* and its further colonization and development as biofilms (i.e., the sessile phase) leads to many oral infections such as denture stomatitis.[4] However, only a few have investigated this phenomenon and the susceptibility of these sessile cells to commonly used antifungals and naturally occurring antimicrobial agents in biological fluids such as saliva.[23–27]

In vivo, *Candida* biofilm studies on denture acrylic are severely restricted due to the complex oral environment. Therefore researchers have evaluated for many years *Candida* adhesion and biofilm development and their antifungal

Table 1 – The metabolic activity of *Candida* biofilms as determined by the XTT-reduction assay and the percentage survival of biofilms treated with antifungals combined with 100 μg/ml lysozyme (compared with untreated biofilms; Control I and, biofilms treated with 100 μg/ml lysozyme; Control II).

	Control I untreated	Control II 100 μg/ml Lz	100 μg/ml Lz + (×MIC) antifungal					
			6	12	18	24	48	96
Nys								
Mean (XTT value)	0.95	0.65	0.32	0.20	0.17	0.14		
S.D.	(0.05)	(0.10)	(0.08)	(0.06)	(0.02)	(0.02)		
p-Value (comparison to Control I)		***	***	***	***	***		
p-Value (comparison to Control II)			***	***	***	***		
% survival of biofilm cells in comparison to Control I	100.0	68.7	33.3	20.7	18.1	15.1		
% survival of biofilm cells in comparison to Control II		100.0	49.8	31.5	26.9	22.4		
AmB								
Mean (XTT value)	0.66	0.34	0.27	0.19	0.18	0.13		
S.D.	(0.09)	(0.11)	(0.09)	(0.04)	(0.04)	(0.03)		
p-Value (comparison to Control I)		***	***	***	***	***		
p-Value (comparison to Control II)			ns	**	***	***		
% survival of biofilm cells in comparison to Control I	100.0	50.9	41.1	29.9	27.1	19.8		
% survival of biofilm cells in comparison to Control II		100.0	89.8	64.7	58.6	41.4		
Keto								
Mean (XTT value)	0.69	0.34		0.22		0.17	0.19	0.17
S.D.	(0.12)	(0.11)		(0.06)		(0.03)	(0.03)	(0.03)
p-Value (comparison to Control I)		***		***		***	***	***
p-Value (comparison to Control II)				**		***	***	***
% survival of biofilm cells in comparison to Control I	100.0	49.6		32.4		25.8	27.5	24.6
% survival of biofilm cells in comparison to Control II		100.0		70.3		56.9	61.4	53.8
5FC								
Mean (XTT value)	0.61	0.34	0.35	0.37	0.39	0.36		
S.D.	(0.17)	(0.07)	(0.10)	(0.08)	(0.09)	(0.14)		
p-Value (comparison to Control I)		***						
p-Value (comparison to Control II)			ns	ns	ns	ns		
% survival of biofilm cells in comparison to Control I	100.0	58.8	58.2	62.6	65.4	59.9		
% survival of biofilm cells in comparison to Control II		–	–	–	–	–		

** $p < 0.01$.
*** $p < 0.001$.

Table 2 – The metabolic activity of *Candida* biofilms as determined by the XTT-reduction assay and the percentage survival of biofilms treated with antifungals/ with and without lysozyme (compared with untreated biofilms; Control I and biofilms treated with 100 μg/ml lysozyme; Control II).

	Control I untreated	Control II 100 μg/ml Lz	Nys	Lz + Nys	5FC	Lz + 5FC
Mean (XTT value)	0.85	0.55	0.24	0.16	0.80	0.61
S.D.	(0.07)	(0.14)	(0.07)	(0.07)	(0.13)	(0.10)
p-value (comparison to Control I)		***	***	***	ns	**
p-value (comparison to Control II)			***	***	*	ns
% survival of biofilm cells in comparison to Control I	100.0	64.0	29.1	18.6	93.9	72.4
% survival of biofilm cells in comparison to Control II		100.0	47.8	32.3	100.0	100.0
% survival of *C. albicans* as determined with CSLM			46.7	16.7	99.3	60.9

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

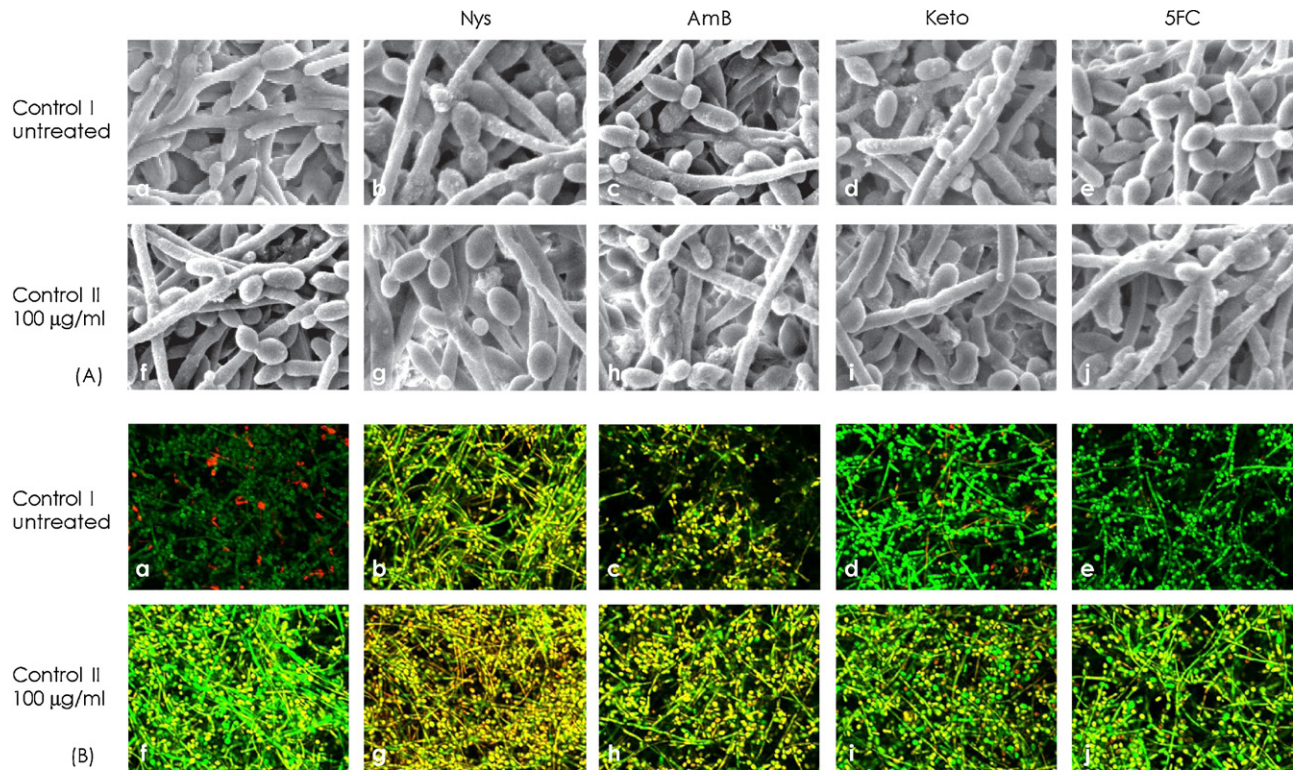

Fig. 6 – (A) SEM visualization of 48 h *C. albicans* biofilms on denture acrylic discs treated with antifungal agents and lysozyme. (a) Untreated 48 h *C. albicans* biofilms (control I), (b–e) 48 h *C. albicans* biofilms treated with ×12 MIC concentrations of nystatin, amphotericin B, ketoconazole and 5-fluorocytosine, (f) 48 h *C. albicans* biofilms treated with 100 μg/ml lysozyme, (g–j) 48 h *C. albicans* biofilms treated with 100 μg/ml lysozyme combined with ×12 MIC concentrations of nystatin, amphotericin B, ketoconazole and 5-fluorocytosine. (B) Confocal scanning laser microscopic of 48 h *C. albicans* biofilms on denture acrylic discs treated with antifungal agents and lysozyme highlighting the live (green) and dead (red) sessile cells stained with LIVE/DEAD BacLight Bacterial Viability Kit (a) untreated 48 h *C. albicans* biofilms (control I), (b–e) 48 h *C. albicans* biofilms treated with (×12 MIC) nystatin, amphotericin B, ketoconazole and 5-fluorocytosine, (f) 48 h *C. albicans* biofilms treated with 100 μg/ml lysozyme, (g–j) 48 h *C. albicans* biofilms treated with 100 μg/ml lysozyme combined with (×12 MIC) nystatin, amphotericin B, ketoconazole and 5-fluorocytosine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

susceptibility *in vitro* on heat or cold-cured acrylic resin strips obtained from cutting out acrylic sheets that have a smooth surface topography which does not represent well the denture surface '*in vivo*'.[28,29] Furthermore the preparation of these acrylic discs in various laboratories have not been standardized making it impossible to compare the results obtained from different research groups. Scanning electron microscopy of these studies have shown that *Candida* in these biofilms are embedded within a matrix of extracellular polymeric material and display microcolonies containing yeasts, hyphae and pseudohyphae arranged in a bi-layer structure.[23,24] However similar visualization of *Candida* biofilms obtained from denture samples from patients with denture stomatitis have shown yeast and filamentous forms deeply embedded within the cracks and irregularities of the acrylic denture.[9] It is this altered phenotype that is believed to be responsible for the distinct properties shown by sessile cells in *Candida* biofilms, most notably their increased resistance to antimicrobial agents.[9,23,24,30]

It is now known that biofilm related infections are unique to the device under consideration and the surface morphology is a very important parameter of plaque formation.[31] Yamauchi et al.[32] have shown that *C. albicans* adherent on acrylic resin with a rough surface was more difficult to dislodge, with ultrasonic cleaning for 1 min than those on a smooth surface. A more recent study by Raab et al.[33] observed that dentures have to be placed in an ultrasonic bath for 90 s for denture plaque to be dislodged.

Taking into consideration the foregoing, acrylic discs used in the present study were prepared with a surface texture that closely resembled that of the denture appliance so as to make the investigation more clinically realistic. The surface topography of these especially designed acrylic discs were quite similar to the palatal denture acrylic surface topography. Furthermore, in pilot experiments we noted that the rotary disc device previously used for bacterial biofilm development[19] fitted with especially designed denture acrylic discs, can be employed to develop standardized *Candida* biofilms with no significant differences between biofilms on individual discs (unpublished data). This system yielded robust biofilm growth with multiple layers and water channels that are characteristic and well described in bacterial biofilms [34]

(Fig. 1). This methodology could hence be valuable for future investigators testing on 48 h *Candida* biofilms.

Lysozyme is an antimicrobial protein found in saliva with a physiological level of 1–57 μg/ml.[35] Its pronounced antifungal activity against the planktonic form of a number of *Candida* species is well known and has been described by our group as well as others.[12,36,37] The results of the present study has shown that during the early adhesion phase (90 min period), *C. albicans* on denture acrylic coupons immersed in a nutrient medium incorporated with varying concentrations of lysozyme (6.25, 12.5, 25.0, 50 and 100 μg/ml) does not show significant differences in metabolic activity (as observed by XTT measurements). This was visually confirmed in separate experiments by scanning electron microscopy when *Candida* blastospores were seen attached to the acrylic surface but without multilayer biofilm development. Therefore it can be hypothesized that even relatively small physiological concentrations of lysozyme in saliva may hamper candidal colonization on denture surfaces. A similar antimicrobial effect for lactoferrin has been reported by Singh et al.[38] who found that although 20 μg/ml lactoferrin kept *Candida* biofilm formation in continuous-culture-flow-cell surfaces it was powerless to remove them when administered after the development of the polysaccharide matrix in the mature biofilm. In a similar vein, we also noted that although a concentration of 100 μg/ml lysozyme showed approximately 45% antifungal activity against 48 h sessile *Candida* cells, further increasing concentrations of lysozyme (upto 240 μg/ml) was ineffective in complete eradication of the mature 48 h biofilm.

Since we observed a marked fungicidal activity for a standard concentration of 100 μg/ml lysozyme against sessile *Candida* cells we monitored the effect of varying concentrations of commonly used antifungals in association with 100 μg/ml lysozyme on *Candida* biofilms. The results indicated that nystatin, amphotericin B and ketoconazole in association with 100 μg/ml lysozyme exhibited an effective synergistic killing effect against *Candida* biofilms in comparison to either 100 μg/ml lysozyme or the antifungal alone.

However 5-fluorcytosine did not exhibit significant differences in susceptibility with or without the addition of 100 μg/ml lysozyme. The failure of 5-fluorocytosine concentrations greatly in excess of the minimum inhibitory concentrations in reducing biofilm cells formed on substrates such as cellulose acetate filters[39] and polyvinyl chloride,[40] have been reported earlier. Although no confirmatory evidence is available it is thought that such resistance is due to the relatively short time the biofilm cells are in contact with the antifungal (250 min) as well as the fungistatic nature of the drug.[39]

The above results were complimented by ultrastructural studies which demonstrated the most damage to the biofilm ultrastructure in sessile cells treated with nystatin and amphotericin B in association with lysozyme. Ruptured blastospores, hyphae and a vastly disrupted biofilm ultrastructure was evident in the latter samples when compared with the drug or lysozyme free controls. The percentage viability results obtained with CSLM of *Candida* biofilms treated with antifungals/lysozyme also were similar demonstrating that in association with lysozyme, nystatin and amphotericin B individually synergise killing of biofilm *Candida*.

The mechanisms by which *Candida* biofilms resist inhibitory effects of antifungal agents are complex.[41,42] The factors contributing to antifungal resistance in *Candida* biofilms on polymethylmethacrylate have not been explained fully and earlier reports have shown that factors such as the biofilm growth rate and the biofilm ultrastructure interfere with antimicrobial therapy.[39,43] It has also been demonstrated that sessile *C. albicans* cells grown on different substrates (i.e., silicone elastomer, polystyrene, denture acrylic, polyvinylchloride and cellulose acetate filters) display variable resistance to amphotericin B, fluconazole, other azole derivatives and 5-fluorocytosine.[24,30,39,40,44] These results while confirming that sessile *Candida* were highly resistant to conventional antifungal agents also indicate that the extent of matrix formation and, the biofilm ultrastructure played a minor role in excluding these drugs from the biofilm. The increase in metabolic activity with biofilm development and maturation and increased expression of drug resistance genes such CDR1, CDR2 and MDR are thought to be likely causes for the increased drug resistance to antimicrobials.[45] Taken together, it seems likely that multiple mechanisms contribute to antifungal resistance of *Candida* biofilm cells.

It is well known that salivary antimicrobial agents play an important role in the maintenance of oral health.[46] Lower levels of salivary volume and secretion rate associated with the elderly,[47] xerostomia patients and individuals with frequent use of drugs that curtail salivary flow [48,49] may be a factor that contributes to frequent episodes of candidal infection in these patients. On the contrary however, higher levels of lysozyme has been observed in saliva of patients with recurrent oral candidosis than in uninfected controls.[50]

In clinical terms, the *in vitro* data presented here confirm that naturally occurring salivary lysozyme may have an important role in preventing *Candida* colonization on denture acrylic surfaces. Our data also indicate synergistic inhibition of biofilms when lysozyme is combined with polyene antifungal agents. Such combinations may have translational value in the development of drugs for denture related *Candida* infections. Furthermore we have established a versatile clinically relevant denture acrylic assay model using the rotary-disc-incubator, suitable for development of standardized *Candida* biofilms over a period of 2 days. Due to the toxicity and the expenditure in manufacturing 'newer' antifungal drugs which are less toxic for clinical use, it is vitally important to find new pharmacological agents that are natural and less toxic and our results indicate that agents found in biological fluids such as lysozyme could be used for this purpose as adjunctive agents to eradicate *Candida* infection. Further studies are however required to evaluate the optimal conditions that are necessary for '*in vivo*' experimentation with such therapeutic agents.

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