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In Vitro Biofilm Activity of Non-*Candida albicans Candida* Species

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Abstract Candidosis has been attributed to *C. albicans*; however, infections caused by non-*Candida albicans Candida* (NCAC) species are increasingly being recognised. The ability of *Candida* to grow as a biofilm is an important feature that promotes both infection and persistence in the host. The biofilms' activity is significant since high activity might be associated with enhanced expression of putative virulence factors, whilst in contrast low activity has previously been suggested as a mechanism for resistance of biofilm cells to antimicrobials. The aim of this study was to determine the metabolic activity of in vitro biofilms formed by different clinical isolates of NCAC species. The in situ total metabolic activity of *C. parapsilosis*, *C. tropicalis* and *C. glabrata* biofilms was determined using 2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay, and the number of cultivable cells was also established by CFU (colony forming unit) counts. The biofilm structure was assessed by scanning electron microscopy (SEM). Results showed that total biofilm metabolic activity was species and strain dependent. *C. glabrata* exhibited the lowest biofilm metabolic activity despite having the highest number of biofilm cultivable cells. Similarly, the metabolic activity of resuspended *C. glabrata* biofilm and planktonic cells was lower than that of the other species. This study demonstrates the existence of intrinsic activity differences amongst NCAC species, which could have important implications in terms of species relative virulence. Furthermore, the absence of an obvious correlation, between cultivable cells number and total biofilm activity, raises the question about which parameter is the most appropriate for the in vitro assessment of biofilms and their potential clinical significance.

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Introduction

Candidosis is the most prevalent opportunistic fungal infection of humans and of particular significance into patients undergoing treatment for cancer [17], organ transplantation [10] and receiving broad-spectrum antibiotics [26]. Candidosis is also one of the most common and persistent infections in HIV-infected individuals and AIDS patients [27].

Amongst *Candida* species, *Candida albicans* is the most commonly isolated and responsible for the majority of superficial and systemic infections [23]. However, many non-*Candida albicans Candida* (NCAC) species, such as *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis* have recently emerged as important pathogens in suitably debilitated individuals. This fact is highlighted by recent epidemiology studies reporting that NCAC are currently estimated to be responsible for approximately 60% of fungemia [4, 6].

A major virulence factor of *Candida* is its ability to adapt to a variety of different habitats and the consequent formation of surface-attached microbial communities known as biofilms [7, 8]. *Candida* biofilms can develop on natural host surfaces or on biomaterials used in indwelling medical devices. Importantly, such biofilms are phenotypically distinct from their 'free-living' or planktonic forms, with biofilms exhibiting elevated resistance to host defences and administered antimicrobial agents [3, 11, 27]. The clinical

significance of biofilms is highlighted by recent estimates that over 65% of all hospital infections originate from these microbial communities [21]. As a consequence researchers are now recognising the importance of studying biofilm communities rather than planktonic forms when characterising the pathogenic potential of microorganisms. So far, a variety of methods have been described for both the in vitro production of biofilms and their subsequent characterisation. Indirect methods based on 96-well microtiter plate assays, which allow the simultaneous quantification of yeasts in a large number of biofilm samples, have been recommended due to their simplicity and sensitivity [13, 18]. A frequently used approach to quantify biofilm cells activity has been the reduction assay of the tetrazolium salt 2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT). In this colorimetric method, XTT is reduced to an XTT formazan product by mitochondrial dehydrogenases of metabolically active cells. The resulting colorimetric changes are considered to be proportional to the number of living cells and their metabolic activity [13, 18].

Previous *Candida* biofilm studies have been mainly focussed on *C. albicans* biofilms [2, 5, 13, 15, 16], with very few examining NCAC species [1, 12, 28]. Furthermore, the vast majority of candidal biofilm research has been focussed on the assessment of biofilm development either by biomass determination or by quantifying the number of cells in the biofilm. The activity of the biofilm itself or its cellular composition has rarely been investigated [18]. This oversight is important given the fact that biofilm activity has significance in terms of pathogenicity as it is a likely indicator of growth, production of hydrolytic enzymes and, in the case of reduced activity, of possible resistance to antimicrobial activity. Therefore, considering that the biofilm activity is a requirement in *Candida* infections, the aim of this study was to determine the metabolic activity of in vitro biofilms formed by different clinical isolates of NCAC species.

Materials and Methods

Organisms and Growth Conditions

A total of 18 clinical strains (Table 1) of *C. parapsilosis* ($n = 6$), *C. tropicalis* ($n = 6$) and *C. glabrata* ($n = 6$) that had previously been recovered from oral, vaginal or urinary tract infections were used in this study. Strains isolated from vaginal and urinary tract infections were kindly provided by the Hospital of São Marcos (Braga, Portugal). *Candida tropicalis* strains 12 and 75 (recovered from the vaginal tract) were provided by the University of Maringá (Maringá, Brazil). Oral isolates were originally isolated from patients attending the Clínica dos Congregados (Braga, Portugal). Three reference strains, namely *C. parapsilosis* (ATCC 22019), *C. tropicalis* (ATCC 750) and *C. glabrata* (ATCC 2001) were also included in this study. The identity of all isolates was confirmed using CHROMagar®Candida (CHROMagar, Paris, France) and by PCR sequencing using specific primers (ITS1 and ITS4) targeting the 5.8S ribosomal RNA subunit gene [29].

Before the experiments, all isolates were cultured on Sabouraud Dextrose Agar (SDA; Merck, Germany) for 48 h at 37°C. Cells were then subcultured in Sabouraud Dextrose Broth (SDB; Merck, Germany) for 18 h at 37°C in an orbital shaker (120 rev/min). Cells were then harvested by centrifugation at $3000 \times g$ for 10 min at 4°C and washed twice in phosphate buffer saline (PBS pH 7, 0.1 M). The cell pellets were finally resuspended in SDB, and the cell concentration was adjusted using an improved Neubauer haemocytometer to a standardised level (1×10^7 or 1×10^8 cells/ml) depending on the experiment.

Biofilm Formation

Biofilms were produced in 96-well microtiter plates (Orange Scientific, Braine-l' Alleud, Belgium). Briefly, standardised inocula (200 µl of yeast cell suspension containing 1×10^7 cells/ml in SDB) were placed into selected wells and incubated at 37°C in an orbital shaker incubator at 120 rev/min. After 24 h, an aliquot of 100 µl of SDB medium was removed and an equal volume of fresh SDB added. The microtiter plates were then incubated for further 48 h. Experiments were performed in triplicate and on at least three separate occasions.

Biofilm Characterisation

In Situ Biofilm Metabolic Activity

An XTT reduction assay [13, 14] was used to determine the in situ biofilm metabolic activity of the NCAC strains. After biofilm formation, the culture medium was aspirated and the non-adherent cells were removed by washing the biofilms twice in PBS. A 200 µl aliquot of a solution containing 100 µg/µl of XTT (2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (Sigma–Aldrich, USA) and 10 µg/µl of phenazine methosulphate (PMS) (Sigma–Aldrich, USA) was then added to each pre-washed biofilm and also to the control wells (to measure background XTT levels). The plates were then incubated for 3 h in the dark, at 37°C with agitation (120 rev/min). Colorimetric changes were measured at 490 nm using a microtiter plate reader (Bio-Tek® Synergy HT, Izasa, Portugal) and the absorbance values were standardised per unit area of well (absorbance/cm^2).

Table 1 Non-*Candida albicans Candida* strains used in this study, values of in situ total biofilm activity, relative biofilm activity and metabolic activity of biofilm and planktonic cells

Species	Strains	Origin	In situ total biofilm activity (Abs values/cm^2) ±SD	Relative biofilm activity (Abs values/ 10^8 CFU)	Cellular metabolic activity (Abs values/10^8 cells) ±SD	
					Biofilm cells	Planktonic cells
C. parapsilosis	534638	Urinary tract	0.198 ± 0.01	0.518	0.548 ± 0.08	0.621 ± 0.05
	553877		0.099 ± 0.04	0.199	0.323 ± 0.04	0.474 ± 0.13
	AD	Oral tract	0.297 ± 0.07	0.055	0.387 ± 0.06	0.702 ± 0.19
	AM2		0.337 ± 0.13	0.116	0.587 ± 0.09	0.407 ± 0.09
	491861	Vaginal	0.405 ± 0.02	1.311	0.534 ± 0.11	0.554 ± 0.11
	513143		0.537 ± 0.04	1.677	0.496 ± 0.11	0.669 ± 0.11
	ATCC 22019	Reference	0.454 ± 0.09	1.134	0.221 ± 0.05	0.492 ± 0.05
C. tropicalis	519468	Urinary tract	0.115 ± 0.03	0.295	0.273 ± 0.01	0.702 ± 0.15
	544123		0.179 ± 0.02	1.059	0.283 ± 0.07	0.867 ± 0.07
	AG1	Oral tract	0.322 ± 0.05	1.094	0.345 ± 0.07	0.467 ± 0.04
	T2.2		0.259 ± 0.02	0.922	0.162 ± 0.02	0.585 ± 0.09
	12	Vaginal	0.185 ± 0.01	0.041	0.313 ± 0.02	0.298 ± 0.01
	75		0.137 ± 0.03	3.672	0.376 ± 0.07	0.323 ± 0.01
	ATCC 750	Reference	0.507 ± 0.09	16.80	0.665 ± 0.11	0.688 ± 0.02
C. glabrata	562123	Urinary tract	0.223 ± 0.04	0.006	0.228 ± 0.07	0.281 ± 0.09
	513100		0.129 ± 0.03	0.002	0.100 ± 0.02	0.149 ± 0.01
	D1	Oral tract	0.237 ± 0.09	0.006	0.179 ± 0.03	0.138 ± 0.07
	AE2		0.201 ± 0.08	0.052	0.167 ± 0.02	0.135 ± 0.04
	534784	Vaginal	0.172 ± 0.07	0.049	0.131 ± 0.03	0.079 ± 0.03
	585626		0.118 ± 0.05	0.031	0.084 ± 0.02	0.201 ± 0.06
	ATCC 2001	Reference	0.238 ± 0.06	0.084	0.181 ± 0.05	0.732 ± 0.14

SD standard deviation

Quantification of Biofilm Cultivable Cells

The number of cultivable biofilm cells was determined by counting colony forming units (CFUs) following biofilm cells resuspension. Briefly, biofilms were first washed twice in PBS to remove loosely attached cells and the biofilm was then resuspended by repeated pipetting. Complete removal of the biofilm was confirmed by subsequent crystal violet staining and spectrophotometric reading for inspection of the wells. The resuspended biofilm (500 µl) was vigorously vortexed for 5 min to disrupt the biofilm matrix and serial decimal dilutions (in PBS) were plated onto SDA. Agar plates were incubated for 24 h at 37°C, and the total CFUs per unit area (Log CFU/cm^2) of microtiter plate well were enumerated. Experiments were repeated on three occasions with individual samples evaluated in triplicate.

Biofilm Structure

Biofilm structure was examined by Scanning Electron Microscopy (SEM). Biofilms were formed by seeding 2 ml of the standardised cell suspension (1×10^7 cells/ml in SDB) into 24-well plates (Orange Scientific, Braine-l'Alleud, Belgium) and incubated for 48 h at 37°C and 120 rev/min. After 24 h, 1 ml of SDB medium was removed and an equal volume of fresh SDB added. At 48 h, the medium was aspirated and non-adherent cells removed by washing the biofilms twice with sterile ultra-pure water. Samples were dehydrated with alcohol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air dried for 20 min. Samples were kept in a desiccator until the base of the wells was removed for analysis. Prior to observation, the base of the wells was mounted onto aluminium stubs, sputter-coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

Quantification of Cellular Metabolic Activity

Metabolic Activity of Biofilms Cells

Biofilms were removed from the microtiter plate wells as described previously and the cell concentration adjusted to 1×10^8 cells/ml using an improved Neubauer

haemocytometer. Cells from 1 ml of the standardised cell preparation were harvested by centrifugation (3000×*g*), and the supernatant was discarded. An aliquot of 1 ml of XTT solution (100 μg/μl of XTT and 10 μg/μl of PMS) was added to the yeast cells pellet. The mixture was then incubated in the dark for 3 h at 37°C and 120 rev/min. Colorimetric changes were measured as previously described and activity expressed as absorbance/1 × 10^8 cells. Experiments were repeated on three occasions with individual samples evaluated in triplicate.

Metabolic Activity of Planktonic Cells

Cells were inoculated in SDB for 18 h at 37°C under agitation (120 rev/min) and then harvested by centrifugation (3000×*g* for 10 min at 4°C) and washed twice in PBS. After resuspension in PBS, the cell concentration was adjusted to 1 × 10^8 cells/ml and the cellular metabolic activity was measured as previously described for the resuspended biofilm cells.

Statistical Analysis

Results were compared using a one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variance and the Tukey multiple-comparisons test, using SPSS software (SPSS [Statistical Package for the Social Sciences], Inc., Chicago, IL). All tests were performed with a confidence level of 95%.

Results and Discussion

The primary aim of this study was to determine the total in situ biofilm activity of 21 strains of NCAC species using an XTT reduction assay (Fig. 1). It was clearly evident that biofilm metabolic activity was species and strain dependent. Specifically, the overall mean metabolic activity of *C. glabrata* (Abs/cm^2 = 0.19 ± 0.05) biofilms was lower than for *C. parapsilosis* (Abs/cm^2 = 0.33 ± 0.15) and *C. tropicalis* (Abs/cm^2 = 0.24 ± 0.14). Such intra species variation in terms of biofilm formation has previously been demonstrated albeit using parameters other than activity [12, 28]. It was interesting to note that in the case of *C. parapsilosis*, both urinary tract isolates (534638 and 553877) had significantly ($P < 0.05$) lower biofilm metabolic activity (Abs/cm^2 = 0.20 ± 0.01; 0.09 ± 0.04) compared with the other *C. parapsilosis* strains. Whether this finding relates to an inherent difference between strains from distinct pathological origins is unclear, and further investigation involving larger number of strains from these conditions is needed to clarify this point.

There are several possible reasons for the observed species and strain differences in in situ biofilm metabolic activity. A key factor would obviously be the relative number of metabolic active cells within the biofilm itself. In order to analyse this, CFU counts for each biofilm were determined. In contrast to what would perhaps be expected, there was no correlation between biofilm metabolic activity (Fig. 1) and CFU number (Fig. 2). It was evident that despite *C. glabrata* biofilms having the lowest metabolic activity, these biofilms had significantly ($P > 0.05$) higher number of cultivable cells per unit area (Log CFU/cm^2 = 9.16 ± 0.59) when compared with *C. tropicalis* (Log CFU/cm^2 = 7.34 ± 0.72) and *C. parapsilosis* (Log CFU/cm^2 = 7.82 ± 0.09). Furthermore, also the intra-strain variation observed for *C. parapsilosis* and *C. tropicalis* in terms of biofilm metabolic activity (Fig. 1) was not associated with differences in CFU numbers (Fig. 2). The observed discrepancy between in situ total biofilm activity and the number of cultivable cells does have important implications for biofilm studies, since it is often assumed

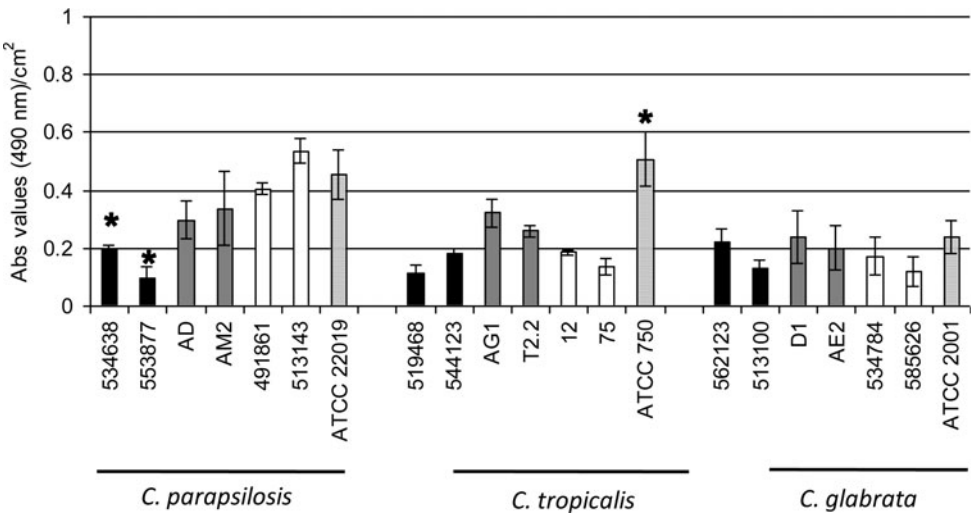

Fig. 1 Absorbance values of XTT solutions per cm^2 of biofilms formed in SDB for 48 h by different clinical isolates of *C. parapsilosis*, *C. tropicalis* and *C. glabrata*. *Error bars* represent standard deviation. * Strains of the same species that are significantly different ($P < 0.05$)

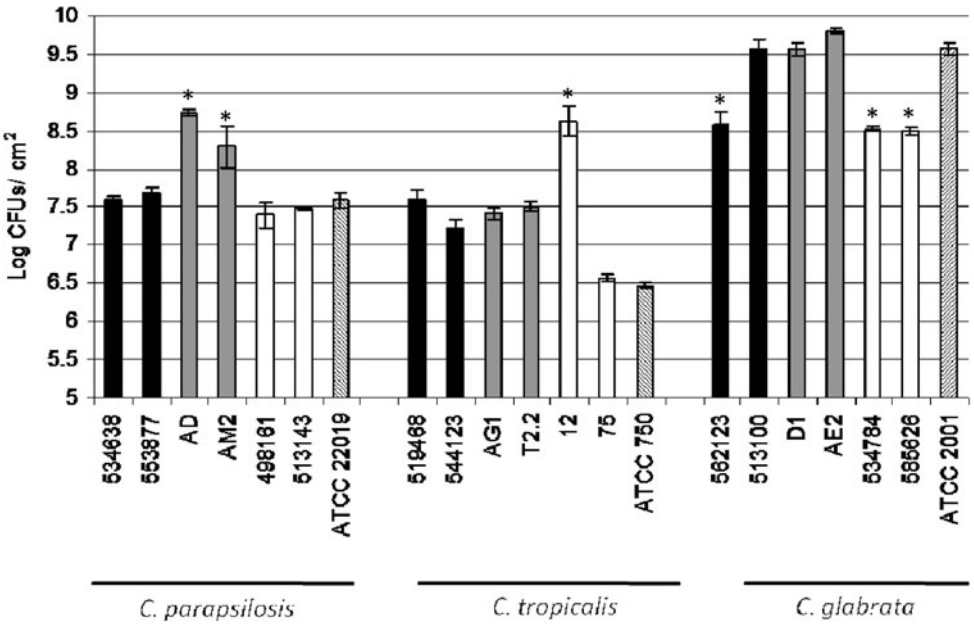

Fig. 2 Logarithm of number cells of *C. parapsilosis*, *C. tropicalis* and *C. glabrata* per cm^2 of the biofilm formed in SDB after 48 h. *Error bars* represent standard deviation. * Strains of the same species that are significantly different ($P < 0.05$)

that metabolic activity measurements provide a sufficient correlation to indirectly quantify biofilms [5, 13, 30]. This situation may be adequate when comparing a single strain type under different environmental conditions but is problematic when multiple strains and species are being compared [19].

In terms of species and strain differences, consideration has to be given to inherent differences in the relative size, morphology and biochemistry of cells. It is known that *C. glabrata* cells are generally smaller (1–4 μm) than *C. tropicalis* (4–8 μm) and *C. parapsilosis* (2.5–4 μm) [24] and also have a narrower spectrum of carbohydrate utilisation [9, 20]. Unlike *C. parapsilosis* and *C. tropicalis*, *C. glabrata* (Fig. 3) is unable to generate filamentous forms which may also be expected to exhibit different metabolic activity, and thus contribute to species differences [18]. Inherent species differences would be supported by the control experiments (Table 1) where planktonic cultures illustrated similar differences between the species with *C. glabrata* again having lower cellular metabolic activity compared with the other two species. Specifically, it was shown that *C. glabrata* biofilm cells had, on average, a lower metabolic activity (Abs/1 $\times$ 10^8 cells $= 0.15 \pm 0.03$) than either *C. tropicalis* (Abs/1 $\times$ 10^8 cells $= 0.35 \pm 0.16$) or *C. parapsilosis* (Abs/1 $\times$ 10^8 cells $= 0.45 \pm 0.15$) cells (Table 1). There were, however, some discrepancies when comparing the relative strain hierarchy for metabolic activity for the planktonic and biofilm cultures. A possible explanation for this relates to potential additional significant phenotypic differences occurring between the two lifestyles [22, 25]. Perhaps the most important of these are those factors that relate to the promotion, persistence and virulence of the organisms within the host environment. As biofilm cells are organised into structured communities embedded within an extracellular matrix, activity within biofilms would be dependent on nutrient access and availability of oxygen, together with removal of waste products. These factors may vary because of inherent differences in the biofilms produced by the tested strains and species, with the resulting alteration in cellular and biofilm metabolic activity. Indeed, evidences of variation in biofilm spatial arrangement are possible observed in Fig. 3. For instances (Fig. 3b), concerning *C. tropicalis* 12, which possess high number of CFU (Fig. 2) and low activity (Fig. 1), it is possible to verify that its biofilm is more cohesive than *C. tropicalis* 75, which presents lower CFU number (Fig. 2) as well as metabolic activity (Fig. 1). The same was observed for *C. glabrata* (Fig. 3a) and *C. parapsilosis* (Fig. 3c) biofilms. Thus, an effect on the ability of XTT to diffuse into these 'different' biofilms is an aspect that also cannot be disregarded. When total biofilm activity was normalised against 1×10^8 CFU of resuspended biofilm (Table 1), it was evident that the relative biofilm activity of *C. glabrata* was 100 times less than *C. tropicalis* and 10 times lower than *C. parapsilosis*. Interestingly, this analysis highlights additional heterogeneity in terms of relative biofilm metabolic activity between *C. parapsilosis* and *C. tropicalis*. Furthermore, the results indicate that, in general, biofilms formed by clinical isolates had a much lower relative activity compared to the corresponding reference strains, with the exception of *C. parapsilosis* ATCC 22019.

In summary, this study underlines both species and strain differences in biofilm metabolic activity which in

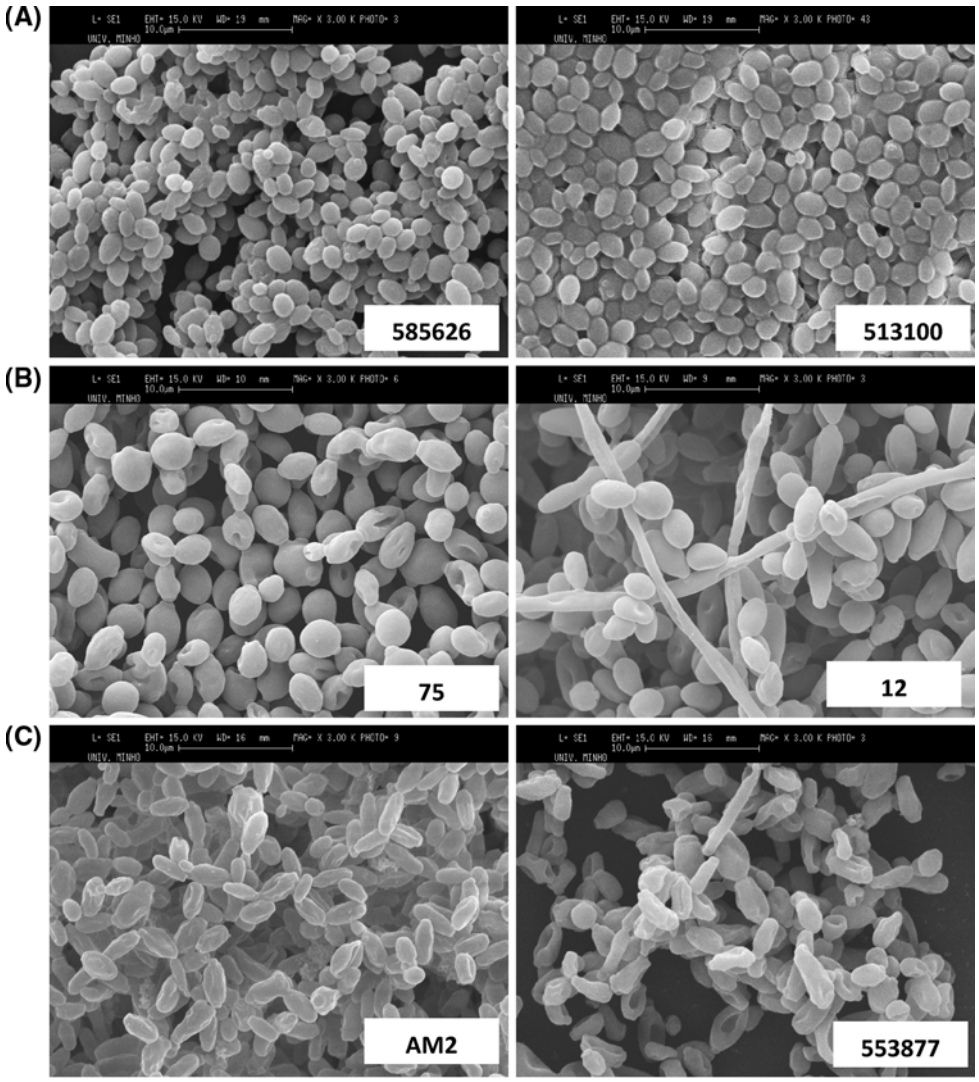

Fig. 3 Biofilm scanning electron microscopy images of two *C. glabrata* (**a**), *C. tropicalis* (**b**) and *C. parapsilosis* (**c**) strains formed in SDB for 48 h. Magnification 3000×, bar 10 μm

part reflects the inherent physiological differences between the organisms. However, it has also to be considered that intrinsic biofilm factors may have a direct effect on the responses obtained. These findings may have significance concerning the pathogenic potential of the strains tested when extrapolated to in vivo situations, with the clinical isolates generally having a lower activity than the equivalent reference species. Such a relatively low activity could promote persistence of the isolates within a clinical environment once the biofilm has become established. Another important aspect raised by this study is that future research needs to consider which is the most appropriate parameter to investigate in vitro biofilm models since total biofilm activity or cultivable cells number do not necessarily reflect the behaviour of the biofilm itself.

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