Candida albicans and *Candida dubliniensis*: comparison of biofilm formation in terms of biomass and activity

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ABSTRACT

Candida albicans and *C. dubliniensis* are two species responsible for oral candidiasis, especially in immunocompromised patients. Microbial infection is preceded by adherence and biofilm formation. Biofilm formation represents the most common form of *C. albicans* in the oral cavity and is considered to be one of the most important virulence factors. In this study, the biofilm formation ability of *C. dubliniensis* was compared with that of *C. albicans* in terms of biomass (quantified using crystal violet) and activity (assessed by formazan salts formation). Both species formed heterogeneous biofilms; however, species and strain variations were seen in the quantification of biomass and activity. There was no correlation between pseudohyphae formation and biofilm formation capability.

KEY WORDS: Biofilms. Candida albicans. Candida dubliniensis.

Introduction

Candida albicans is one of the most well-known *Candida* species, as it is responsible for the majority of infections with such organisms. More recently, another species, *C. dubliniensis*, which is closely related to *C. albicans*, was recovered from the oral cavity of human immunodeficiency virus (HIV)-infected patients.[1] Furthermore, presence of this species in cases of superficial and systemic disease in immunocompromised individuals has been reported.[2,3]

As *C. dubliniensis* is only rarely recovered from the oral cavity of healthy individuals, its prevalence in HIV-infected and acquired immune deficiency syndrome (AIDS) patients indicates that it is an opportunistic pathogen in such immunocompromised states, probably from the patient's own microbial flora. In this environment, the ability of *C. dubliniensis* to adhere strongly to oral epithelial cells may provide it with a competitive advantage over other, less-adherent non-albicans *Candida* species.[4]

One of the most important virulence factors of *Candida* species is their ability to form biofilms, and thus they assume greater significance in the clinical context because they are associated with a significantly enhanced ability to express resistance against most antimicrobial agents.[5,6] Candidiasis is often an implant-related infection in which adherent microbial populations are found on the surfaces of indwelling devices such as catheters, prosthetic heart valves and joint replacements.[7]

Biofilm infections can be caused by a single microbial species or by a mixture of bacterial and fungal species.[8,9] Biofilms are notoriously difficult to eliminate and are a source of many resistant infections.[10,11] Biofilm formation has been largely studied in relation to the effect of antifungal agents.[12–14]

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Although fungal infection-associated biofilm formation is less common than the bacterial variety, the former tends to be more serious.[15] Therefore, many studies have focused on the *C. albicans* biofilm, due to its well-recognised virulence, whereas only a few studies of the *C. dubliniensis* biofilms have been reported.[16–18]

In a previous study by this group, strains of *C. albicans* and *C. dubliniensis* were shown to be equally capable of colonising the oral cavity, and that the presence of saliva enhances candidal colonisation.[19] The present study aims to evaluate the ability of each species to form a biofilm in the oral environment.

Materials and methods

Cells were grown in Sabouraud dextrose broth (SDB, Merck), which is the most common medium used to grow *Candida* species, or in an artificial saliva growth medium.

Sabouraud dextrose medium

Yeast cells were maintained in Sabouraud dextrose agar (SDA) that was prepared according to the manufacturer's instructions (30 g/L) plus 1.7 % agar (Merck). Sabouraud dextrose broth (SDB, Merck; 30 g/L in water) was used to grow the cells in liquid medium.

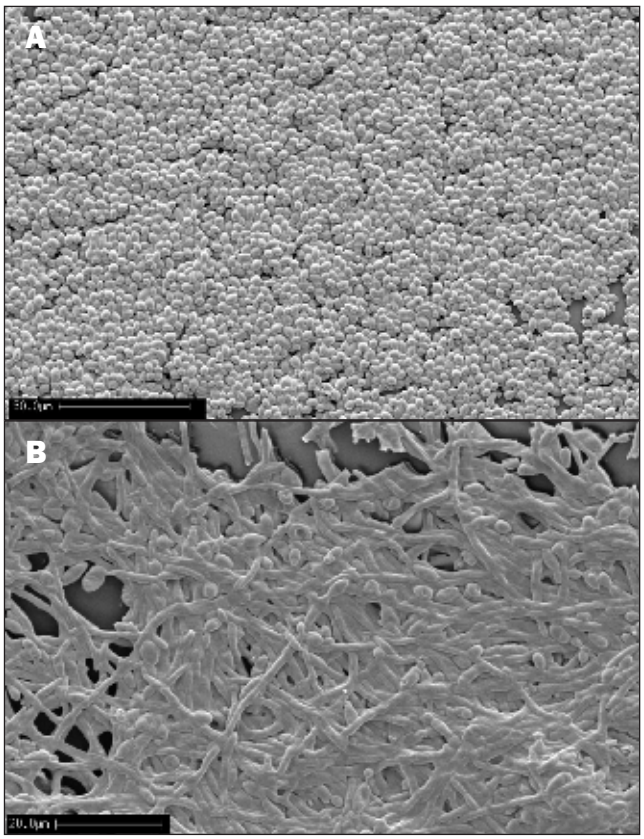

Fig. 1. SEM observation of a general view of a mature biofilm of *C. albicans* 12A at a) x500 (bar corresponds to 50 μm) and b) x1000, containing blastopores, hyphae and pseudohyphae (bar corresponds to 20 μm).

Artificial saliva medium
In some experiments artificial saliva was used to mimic in vivo oral conditions. This was prepared according to Gal *et al.*[20] and comprised 125.6 mg/L NaCl, 963.9 mg/L KCl, 189.2 mg/L KSCN, 654.5 mg/L KH_2PO_4, 200 mg/L Urea, 763.2 mg/L $Na_2SO_4.10H_2O$, 178 mg/L NH_4Cl, 227.8 mg/L $CaCl_2.2H_2O$ and 630.8 mg/L $NaHCO_3$. In order to create an artificial saliva growth medium, glucose (2 g/L), yeast extract (2 g/L) and peptone (5 g/L) were added to the solution.[21] pH was adjusted with CO_2 to 6.8.

Yeast cells
The *Candida* species used were *C. albicans* and *C. dubliniensis* and two different strains of each were assayed. For *C. albicans* one strain was from American Type Culture Collection, ATCC 32354 (*C. albicans* B311) and the other was a clinical isolate (*C. albicans* 12A). For *C. dubliniensis* two strains were obtained from CBS (*C. dubliniensis* 7987 and *C. dubliniensis* 7988). For all assays, yeast cells were grown for 24 h in SDA at 37^{0}C. The cells were then inoculated in SDB for 18 h at 37^{0}C and 150 rpm. After incubation the cells were harvested by centrifugation for 10 min at 5000 rpm and 4^{0}C.

Acrylic surfaces
Acrylic coupons were prepared as described by Samaranayake and MacFarlane.[22,23] Briefly, 1.5 g self-polymerising acrylic powder was mixed with 1 ml monomer in liquid phase. After mixing, the solution was poured onto a surface covered with aluminium foil. After 45 sec another surface was placed on top of the polymerising mixture. After 30 min the acrylic sheet was cut into squares, 8 x 8 mm^2.

Biofilm formation
Yeast cells were subcultured in SDA for 24 h at 37^{0}C, followed by growth in SDB at 37^{0}C and 150 rpm for 18 h. Cells were harvested by centrifugation (5000 rpm, 10 min) and resuspended in SDB or in artificial saliva growth medium to 5x10^7 cells/mL. The biofilm was formed on acrylic coupons 24-well plates, with each well containing 1 mL yeast cell suspension. The medium (SDB or artificial saliva growth medium) was replaced by fresh medium every 12 h and biofilm formation was inspected after 7, 14, 24, 48 and 72 h.

Crystal violet
Coupons containing biofilm were removed from each well and immersed for 15 min in wells containing 1 mL methanol. After withdrawing the methanol, the coupons were allowed to dry at room temperature before adding 600 μL crystal violet. After 5 min the coupons were washed in water and immersed in acetic acid (33 %) to dilute the stain. Absorbance was read at 570 nm.

Tetrazolium salts
Coupons containing biofilm were withdrawn from each well and immersed in 1 mL solution of 100 μg/μL XTT and 10 μg/μL PMS. The well plate was incubated in the dark for 3 h with agitation (150 rpm). Each solution was centrifuged for 3 min at 1000 rpm and the absorbance was read at 490 nm.

Statistical analysis
Data were analysed using the SPSS (Statistical Package for the Social Sciences) program. One-way ANOVA with the Bonferroni test was used to compare the number of adherent cells of the four strains. All tests were performed with a confidence level of 95%.

Results

Biofilm quantification was performed using two different approaches: total biomass formed using crystal violet (CV) staining; and quantification of the amount of active cells by the reduction of a tetrazolium salt (XTT). Correlation and significance values obtained for the two strains of *C. albicans* and *C. dubliniens*is are presented in Table 1.

Table 1. Correlation factors (r^2) and significance values *(P)* obtained for the absorbance of CV-stained suspensions of *C. albicans* B311, *C. albicans* 12 A, *C. dubliniensis* 7987 and *C. dubliniensis* 7988 versus the absorbance of the same cell suspensions stained by XTT

Strain	r^2	*P*
Candida albicans B311	0.973	0.014
Candida albicans 12A	0.991	0.000
Candida dubliniensis 7987	0.989	0.000
Candida dubliniensis 7988	0.978	0.001

The values show a statistical significant correlation between the absorbance of both evaluation methods for all the strains studied. Scanning electron microscope (SEM) observations of biofilms formed by *C. albicans* 12A are presented in Figure 1. The images show that the biofilms were thick and heterogeneous, with either the predominance of blastopores or mycelia.

Figures 2 and 3 demonstrate the evolution of the biofilm biomass quantified by crystal violet staining.

In order to aid interpretation of these results the slope between 48 and 72 h was determined and the values presented in Table 2. The slopes of the biofilm formation profiles for *C. albicans* B311 and *C. dubliniensis* 7987 are similar, as is the case with the other two strains.

Biofilm activity profiles formed on either SDB or artificial saliva growth medium are presented in Figures 4 and 5. The slope of the different curves seen between 48 and 72 h was determined and is presented in Table 3. Activity of all biofilms formed in SDB in effect levelled after 48 h. The only profile that does not present a null slope corresponds to *C. dubliniensis* 7987 in artificial saliva.

Table 4 presents the significance values obtained by both quantification methods (CV and XTT). Significant differences were seen among the biofilms formed in both media (95% confidence level). Comparing the results obtained in terms of biofilm mass and biofilm activity, it can be concluded that cell activity is not dependent on cell number. In the case of a mature biofilm, the number of total cells might be high but their activity can be low. For instances, the cells in the deeper layer may be less active as a result of diffusional limitations.

Presence of hyphae at 48 h was observed under SEM (Figs. 6 and 7). All the biofilms were compact and it is possible to see some pseudohyphae. *C. albicans* B311 formed biofilms with more and longer pseudohyphae, both in SDB and in artificial saliva. All other strains formed biofilms that produced pseudohyphae only in artificial saliva growth medium.

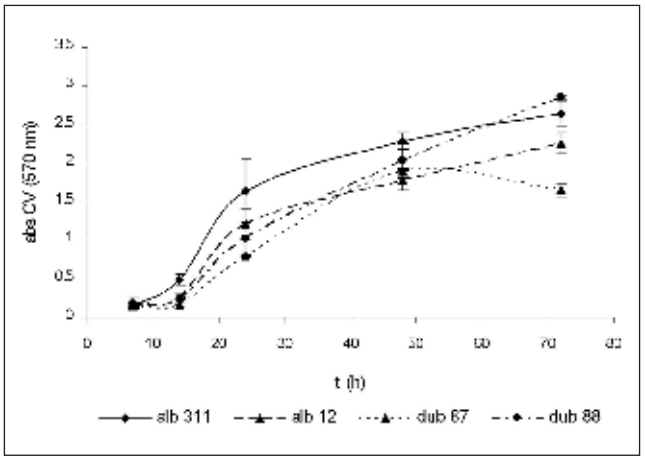

Fig. 2. Absorbance values of crystal violet solutions obtained from biofilms formed in SDB after 7, 14, 24, 48 and 72h by *C. albicans* B311 (alb 311), *C. albicans* 12 A (alb12), *C. dubliniensis* 7987 (dub 87) and *C. dubliniensis* 7988 (dub 88).

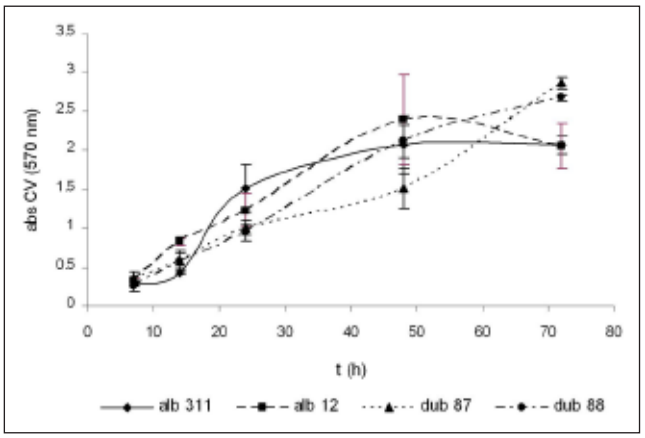

Fig. 3. Absorbance values of crystal violet solutions obtained from biofilms formed in artificial saliva after 7, 14, 24, 48 and 72 h by *C. albicans* B311 (alb 311), *C. albicans* 12 A (alb12), *C dubliniensis* 7987 (dub 87) and *C. dubliniensis* 7988 (dub 88).

Discussion

Candida spp. biofilms are highly heterogeneous. This mixture of yeast blastopores, hyphae and matrix material is not seen when the organism is grown in liquid culture or on an agar surface, which suggests that morphogenesis is triggered when an organism contacts a surface and that the basal cell layer may have an important role in anchoring the biofilm to the surface.[24–26]

Overall organisation of a candidal biofilm is generally similar to that of a bacterial biofilm, but details of its structure are highly dependent on the conditions under which it is formed. This plasticity in structure suggests that biofilms formed in the human host may also vary depending on the nature of the implanted device and in location.[27] Variables such as surface material,[28] medium,[29] presence of other microorganisms,[30] incubation conditions[26] and cell density[31] can influence the structure of the resulting biofilm and the morphology of cells within it.

The demonstration that different conditions lead to different morphologies suggests that both morphological forms (yeast cells and hyphal cells) are capable of biofilm formation. To confirm this, Baillie and Douglas[32] showed that a morphological mutant unable to produce hyphae formed a dense biofilm composed of blastopores, whereas a mutant that produced only filamentous cells formed a hyphal biofilm.

Table 2. Values of the slopes of the biofilm mass profiles (between 48 and 72 h) of *C. albicans* B311, *C. albicans* 12A, *C. dubliniensis* 7987 and *C. dubliniensis* 7988 evaluated with CV in both biofilm formation media

	Slope	
Strain	SDB	Artificial saliva growth medium
Candida albicans B311	0.015	0.023
Candida albicans 12A	0.020	0.049
Candida dubliniensis 7987	–0.010	0.021
Candida dubliniensis 7988	0.034	0.049

One of the major conclusions of the present study is the fact that *C. albicans* and *C. dubliniensis* can form mature biofilms, whereas, for instances, *Saccharomyces cerevisae* can adhere but not form biofilm.[26] This is important because it represents one of the pathogenic features of *Candida* species.

Another conclusion is that biofilm formation is either strain or species dependent. *C. dubliniensis* 7987 shows a different behaviour to that of other strain of the same species and the strains of *C. albicans*. Furthermore, *C. albicans* 12A and *C. dubliniensis* 7988 show similar behaviour in almost all conditions studied, with the exception of biofilm biomass formation in artificial saliva. *C. albicans* B311 also shows different behaviour from the other strains. Results on the biofilm activity of *C. dubliniensis* 7987 are similar to those obtained by Ramage *et al*.[16]

One of the goals of this work was the quantification of biofilm characteristics by two different methods (crystal violet and formation of tetrazolium salts [XTT]). The XTT method is usually used in studies on the susceptibility of *Candida* species biofilms to antifungal agents.[33,34] However, Kuhn *et al*.[35] described some limitations of this method. They conclude that it cannot be assumed that there is necessarily a linear relationship between the number of cells and the colorimetric signal, and that the relationship between XTT concentration and the resultant colorimetric signal is not necessarily proportional.

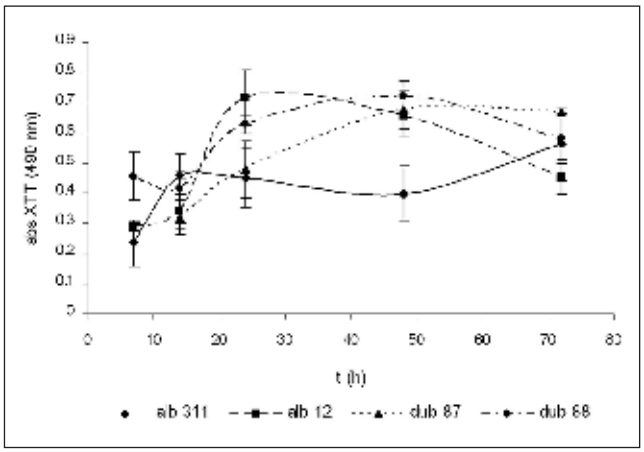

Fig. 4. Absorbance values of XTT solutions obtained from biofilms formed in SDB after 7, 14, 24, 48 and 72 h by *C. albicans* B311 (alb 311), *C. albicans* 12 A (alb 12), *C. dubliniensis* 7987 (dub 87) and *C. dubliniensis* 7988 (dub 88).

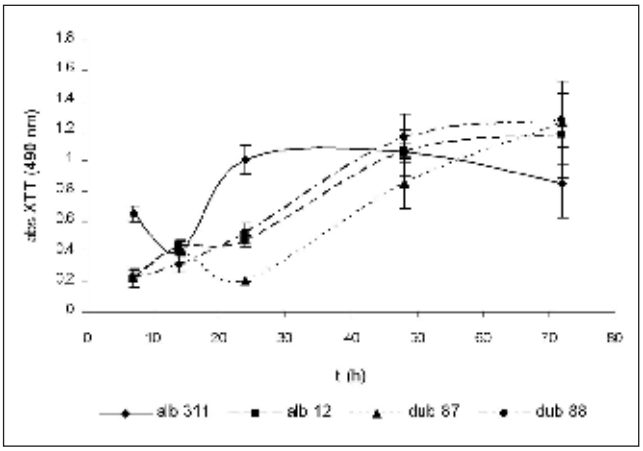

Fig. 5. Absorbance values of XTT solutions obtained from biofilms formed in artificial saliva after 7, 14, 24, 48 and 72 h by *C. albicans* B311 (alb 311), *C. albicans* 12 A (alb 12), *C. dubliniensis* 7987 (dub 87) and *C. dubliniensis* 7988 (dub 88).**Fig. 2.** Description of departmental results.

Table 3. Values of the slopes of the biofilm activity profiles (between 48 and 72 h) of *C. albicans* B311, *C. albicans* 12A, *C. dubliniensis* 7987 and *C. dubliniensis* 7988 evaluated with XTT, in both biofilm formation media.

Strain	Slope	
	SDB	Artificial saliva growth medium
Candida albicans B311	0.007	–0.008
Candida albicans 12A	–0.008	0.004
Candida dubliniensis 7987	–0.000	0.016
Candida dubliniensis 7988	–0.006	0.005

In the present study, the high correlation (Table 1) between absorbance of XTT and CV solutions means that these methods can be applied for cell quantification of suspended cultures at the exponential growth phase. Good correlation between the absorbance of CV and XTT solutions allows the use of both methods to compare differences in cell concentrations.

Yin *et al*.[36] reported that cell activity measured by XTT is associated linearly with the number of cells, confirming the reliability of XTT staining in biofilm quantification. However, as biofilm cells are enclosed in an exopolymeric matrix, this may limit access to nutrients and oxygen, resulting in possible alterations in cellular metabolic activity. If this is the case, the XTT assay, which is based on metabolic activity, may not determine accurately the number of cells. Other method, such as CV staining, should be used to quantify total biomass.

In comparing both methods of biofilm evaluation, it is interesting to note that biomass increases with time while activity decreases. This can be explained by the fact that a biofilm is composed of several cell layers and the basal ones may not be as active as those on the top of the biofilm.

Pseudohyphae formation is considered to be a virulence factor of *Candida* species and one of the determinants in adhesion interactions.[37] The present results (Figs. 6 and 7) demonstrate that artificial saliva promotes the formation of pseudohyphae, but there was no evidence to suggest that pseudohyphae enhance biofilm formation.

Comparing both media, it is interesting to note that *C. dubliniensis* 7987 biofilm formation profiles show a positive and significant slope in artificial saliva, but the opposite happens in SDB. Table 4 shows the significant differences between the media are not constant, as they vary with evaluation method, strain and biofilm age.

Other studies involving the use of artificial saliva resulted in different conclusions. According to Jin *et al*.,[29] the presence of saliva does not significantly influence biofilm formation, while Nikawa *et al*.[38] showed that the presence of saliva increases biofilm formation by *C. albicans*.

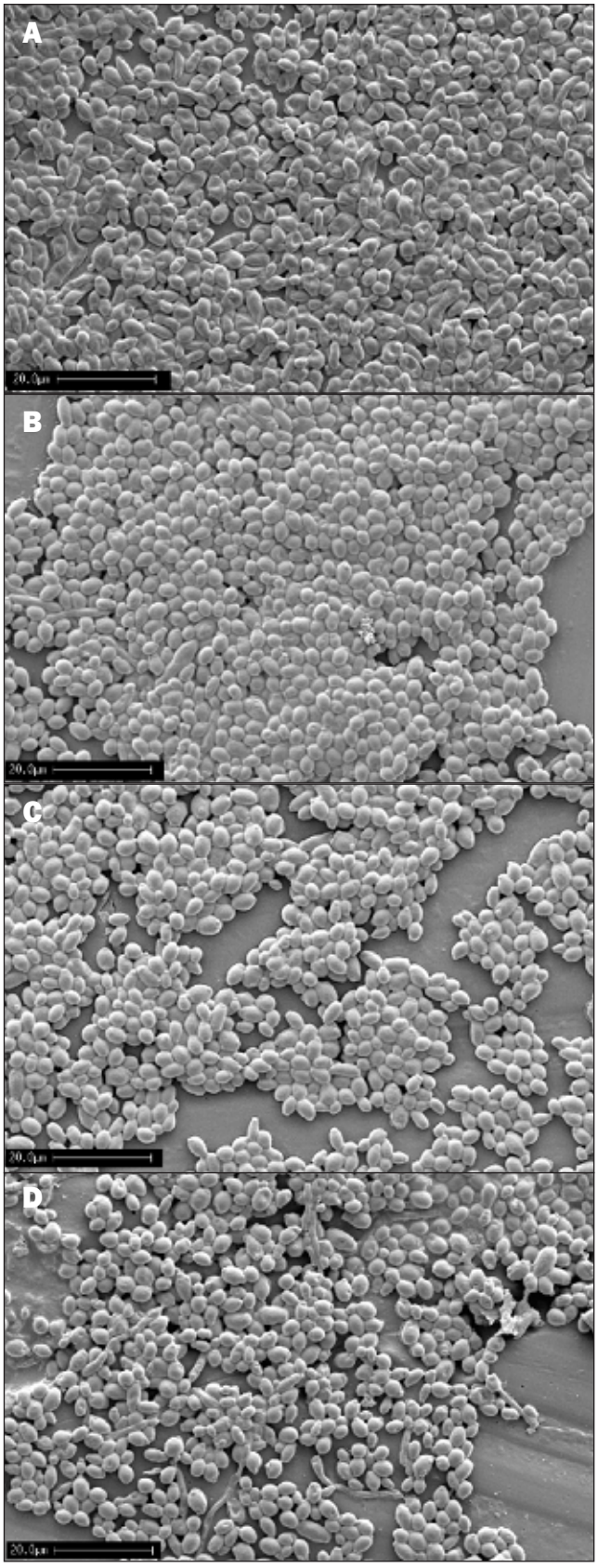

Fig. 6. SEM images of biofilms formed in SDB after 48h for a) *Candida albicans* B311, b) *C. albicans* 12 A, c) *C. dubliniensis* 7987 and d) *C. dubliniensis* 7988 (original magnification x1000; bar represents 20 μm).

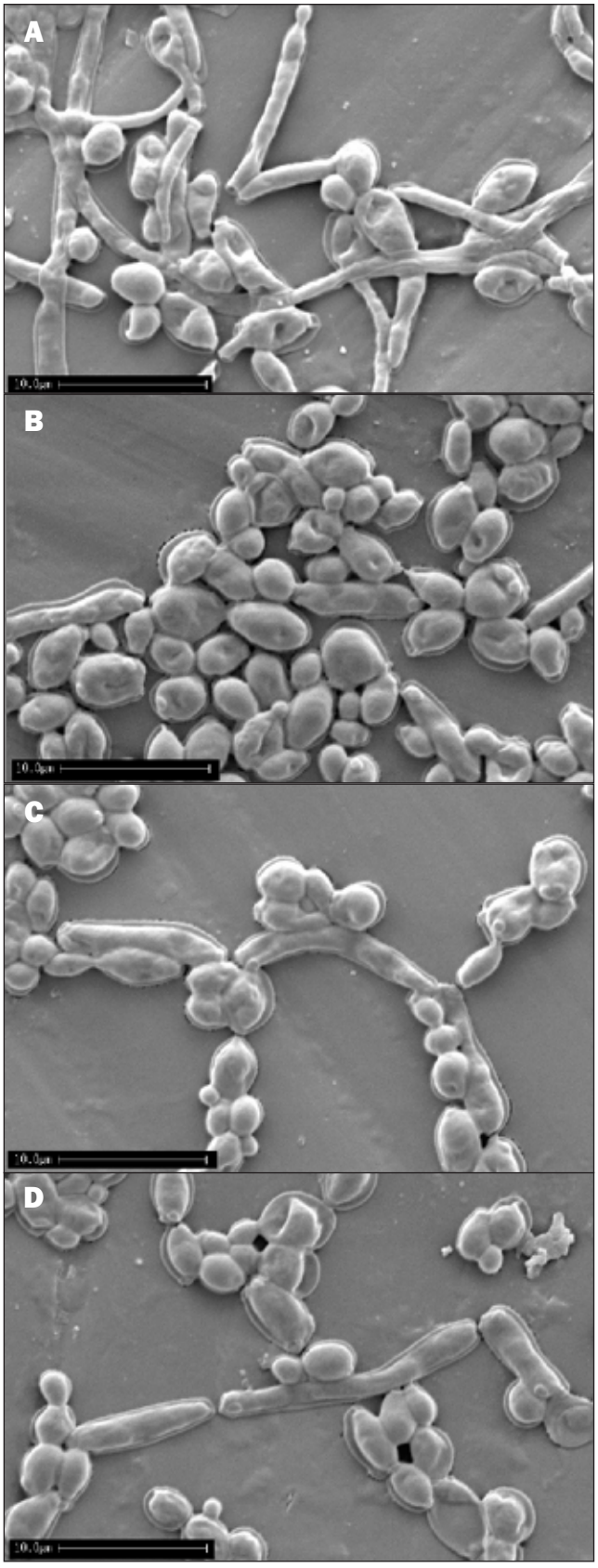

Fig. 7. SEM images of biofilms formed in artificial saliva after 48 h by a) *Candida albicans* B311, b) *C. albicans* 12 A, c) *C. dubliniensis* 7987 and d) *C. dubliniensis* 7988 (original magnification x3000; bar represents 20 μm).

Table 4. Significance values (P) obtained comparing biofilms formed by *C. albicans* B311 (alb 311), *C. albicans* 12 A (alb 12), *C. dubliniensis* 7987 (dub 87) and *C. dubliniensis* 7988 (dub 88) in SDB and artificial saliva growth medium, for both evaluation methods and all times assayed.

	Significance value (*P*)							
	CV				XTT			
Time (h)	alb 311	alb 12	dub 87	dub 88	alb 311	alb 12	dub 87	dub 88
7	0.005	0.009	0.041	0.382	0.009	0.142	0.002	0.016
14	0.229	0.003	0.005	0.006	0.055	0.157	0.325	0.253
24	0.683	0.922	0.460	0.571	0.002	0.038	0.020	0.137
48	0.384	0.279	0.067	0.317	0.003	0.001	0.550	0.034
72	0.007	0.305	0.026	0.481	0.122	0.000	0.001	0.003

It is well known that some salivary proteins, such as human fibronectin and members of the proline-rich protein family, can act as receptors for *C. albicans*. On the one hand, these proteins, when immobilised on a surface, may promote candidal adhesion and subsequent biofilm formation by acting as receptors for free-living planktonic yeasts, while they may simultaneously block binding sites originally present on the substratum. In addition, the presence of salivary antimicrobial proteins has been demonstrated,[39] which further complicates this issue.[29]

In the present study, the influence of saliva on biofilm formation was assessed using artificial saliva growth medium instead of natural saliva. Use of whole saliva has some disadvantages (donor and time of day variations) that can be overcome with an artificial solution.

Adhesion of strains used in the present study to acrylic was studied previously,[19] and results showed that there were no statistical differences among them, either using water or artificial saliva. However, in terms of biofilm formation, *C. dubliniensis* 7987 showed different behaviour to that of *C. albicans* and *C. dubliniensis* 7988, both of which produced similar results. This difference was seen in biomass accumulation and cellular activity for both media (SBD and artificial saliva growth medium).

As a general conclusion, the number of initially adherent cells showed no direct correlation with biofilm evolution. Thus, adhesion and biofilm formation can be considered distinct factors of candidal virulence, and biofilm formation is strain dependent. □

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