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Original article

Variation in biofilm formation among blood and oral isolates of *Candida albicans* and *Candida dubliniensis*

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ABSTRACT

Introduction: Biofilm production is considered a potential virulence factor of some *Candida* species. For this reason, an understanding of biofilm behavior of *Candida albicans* and its closely related species *Candida dubliniensis* is key to the development of effective preventive measures for invasive and oral candidiasis. The aim of this study was to compare the capacity of biofilm production by blood and oral isolates of *C. albicans* and *C. dubliniensis* using polystyrene, flat-bottomed 100-well microtiter plates.
Methods: A total of 47 isolates, consisting of 28 *C. albicans* (16 oral and 12 blood isolates) and 19 *C. dubliniensis* (11 oral and 8 blood isolates) were compared for their biofilm forming ability under aerobic and static conditions. XTT reduction assay was used to quantify the sessile growth.
Results: All tested isolates produced biofilm, measured as XTT metabolic activity. Biofilm formation by *C. albicans* isolates was statistically significantly higher than biofilm formation by *C. dubliniensis* isolates at 24 h ($P = 0.03$) and 48 h ($P = 0.0001$). There was a higher percentage (41.7%) of high producers of biofilms among *C. albicans* blood isolates than among oral isolates (31.3%), without statistically significant differences.
Conclusions: This capability may allow *C. albicans* and *C. dubliniensis* to maintain their oral ecological niches as commensal or pathogenic microorganisms and can be a major virulence factor during invasive candidiasis. However, the differences in biofilm production among isolates should be taken into account when the anti-biofilm activity of antifungal agents or other virulence factors are tested *in vitro*.

Evaluación de la capacidad de formación de biopelícula de aislamientos clínicos de *Candida albicans* y *Candida dubliniensis*

Palabras clave:
Biopelícula
Sangre
Boca
Candida albicans
Candida dubliniensis

RESUMEN

Introducción: El desarrollo de biopelículas o biocapas se considera un factor de virulencia potencial de algunas especies de *Candida*. Una comprensión mejor del comportamiento de las biopelículas de *Candida albicans* y de la especie cercana *Candida dubliniensis* es clave para desarrollar medida preventyivas eficaces de las candidiasis superficiales (orales) e invasoras. El objetivo de este trabajo ha sido comparar la capacidad de producir biopelícula por parte de los aislamientos orales y de sangre de *C. albicans* y *C. dubliniensis* en placas de microtitulación de poliestireno de 100 pocillos con fondo plano.
Métodos: Se estudiaron 47 aislamientos: 28 *C. albicans* (16 orales y 12 hemáticos) y 19 *C. dubliniensis* (11 orales y 8 hemáticos). Se empleó una prueba de cuantificación de la actividad metabólica de las biopelículas (reducción de la sal de tetrazolio denominada XTT).
Resultados: Todos los aislamientos mostraron actividad metabólica pero la formación de biopelícula por los aislamientos de *C. albicans* era significativamente mayor que por los de *C. dubliniensis* a 24 h ($P = 0{,}03$) y 48 h ($P = 0{,}0001$). Eran más numerosos, los aislamientos de *C. albicans* muy productores de biopelícula procedentes de sangre (41.7%) que de boca (31.3%) pero las diferencias no eran significativas.

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Conclusiones: La capacidad de desarrollar biopelículas, podría permitir a *C. albicans* y *C. dubliniensis* mantenerse en el nicho oral como comensales o patógenos y ser un importante factor de virulencia en las candidiasis invasoras. Sin embargo, las diferencias encontradas entre los aislamientos productores de biopelícula deben tenerse en cuenta, sobre todo en los estudios *in vitro* de virulencia o de la acción anti-biopelícula de los fármacos antifúngicos.

Introduction

Invasive candidiasis represents about 10% of nosocomial invasive infections. Although other species, such as *Candida parapsilosis*, *Candida glabrata* or *Candida tropicalis*, are being isolated with increasing frequency; *Candida albicans* is the most frequent aetiological agent of candidiasis.[1,2] Many candidiasis are associated with prostheses, catheters and other indwelling medical devices, where these microorganisms develop biofilms. The presence of extracellular polymers and a different cellular phenotype, called sessile is an important feature in the structure of these microbial comunities. Biofilms impede the actions of the immune system and other defence mechanisms and become recalcitrant to current antifungal treatment.[3–5] *C. albicans* is also an important pathogen of the oral cavity and other mucosae. This yeast has the ability to grow under diverse oral environmental conditions (e.g. unhygienic dentures, xerostomia) and/or systemic factors, such as diabetes and immunodeficiency. The presence of dentures that are overlaid with proteins and other oral components encourages the development of *Candida* biofilms and denture stomatitis.[4]

Candida dubliniensis is an emerging species associated to oral candidiasis, mainly in HIV-infected patients.[6–8] The frequence of deep-seated infections caused by *C. dubliniensis* is low but probably understimated because *C. dubliniensis* is closely related to *C. albicans* and many laboratories are not capable of differentiating between both species.[1,9–13] *C. dubliniensis* shares many properties with *C. albicans* and, in addition, shows an important capacity to develop resistance to fluconazole and other antifungal agents under repeated exposure.[7,14–16]

The aim of the current study has been to compare the capacity of biofilm production by blood and oral isolates of *C. albicans* and *C. dubliniensis* from infected patients.

Methods

Microorganisms

A total of 28 *C. albicans* (16 oral isolates and 12 blood isolates) and 19 *C. dubliniensis* (11 oral isolates and 8 blood isolates) from the Laboratorio de Micología Médica at the University of the Basque Country were studied. The clinical origin of the isolates and the infections caused have been described previously.[8–10,17] Two strains from the National Collection of Pathogenic Fungi (NCPF), *C. albicans* NCPF 3153 and *C. dubliniensis* NCPF 3949, as well as *C. albicans* NCPF 3153 hypha-defective mutant CA-2 (kindly donated by Professor Antonio Cassone, Istituto Superiore di Sanità, Rome, Italy) were included as reference strains. The identity of all isolates was confirmed by conventional mycology methods, such as the germ tube test in serum, microscopic morphology, chlamydoconidia production in corn meal agar with Tween 80, and carbon source assimilation with the commercial kit ID 32 C (bioMérieux, France).[18] All isolates were screened for their ability to grow on Sabouraud dextrose agar at 45 °C for 48 h, and in the chromogenic media CHROM-Pal's medium[19] and ChromID Candida.[20] The reactivity with a specific polyclonal anti-*C. dubliniensis* antibody by an immunofluorescence assay[21] and by the Bichro-Dubli latex agglutination test (Fumouze Diagnostics, France)[22] were also tested. The identities of those isolates classified as *C. dubliniensis* were confirmed by means of a polymerase chain reaction (PCR) using the specific primers CDBF28-f (5′-AAATGGGTTTGGTGCCAAATTA-3′), and CDBR110-r (5′-GTTGGCATTGGCAATAGCTCTA-3′) described by Kanbe et al.[23] which amplify topoisomerase II gene, giving a DNA product size of 816 bp.

Preparation of Candida suspensions

Prior to each experiment, yeast isolates were aerobically cultured at 37 °C for 24 h on Sabouraud dextrose agar (Difco Laboratories, USA) and a loopful of growth was inoculated in YPD broth (yeast extract 10 g, peptone 20 g, glucose 20 g, distilled water 1 l). After 24 h, the yeasts were harvested, washed twice with phosphate buffered saline (PBS, pH 7.2) and suspended to a concentration of 10^7 cells/ml. This cell concentration was selected because previous workers have demonstrated an optimal degree of biofilm formation of *C. albicans* and *C. dubliniensis* at this particular concentration.[24]

Production and quantification of Candida biofilms

Biofilm formation was measured using a colorimetric method based on the XTT reduction according to Ramage et al.[25,26] Briefly, *C. albicans* and *C. dubliniensis* isolates were suspended in RPMI 1640 broth supplemented with L-glutamine and buffered with MOPS, and 100 μl of standard cell suspensions of yeasts (10^7 cells/ml), prepared as above, were transferred into each well of a flat-bottomed 100-well microtitre plate (BioScreen, Finland). XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Sigma, USA) was prepared as a saturated solution in 0.5 g/l in Ringer's lactate and the solution was filter sterilised using a 0.22-μm-pore-size filter (Sarstedt, Germany), aliquoted and then stored at −70 °C until required. Before each assay, an aliquot of stock XTT was thawed and the electron-coupling agent menadione, 10 mM prepared in acetone (Sigma) was added to a final concentration of 1 μM. The biofilms were washed 3 times with 100 μl of PBS to remove loosely adherent cells. Afterwards, 100 μl of the XTT–menadione solution was added to each biofilm and control wells to measure background XTT reduction levels, and the plates were incubated in the dark for 1 h at 37 °C. The colour changes in the XTT reduction assay (which directly correlate with the metabolic activity of the cells within the biofilm) were then measured using a BioScreen C plate incubator (BioScreen) at 492 nm. Measurements were repeated at least six times each on different days. The optical densities (OD) corresponding to the biofilm metabolic activities of both *Candida* species were compared calculating the Student's *t*-test using the program SPSS (IBM, USA). Biofilm production was scored and divided into 6 categories, according to absorbance at 492 nm (A)/transmittance (T), as 6+ ($A > 1.30/T \leq 5\%$), 5+ ($A = 1–1.29/T = 6–10\%$), 4+ ($A = 0.70–0.99/T = 11–20\%$), 3+ ($A = 0.40–0.69/T = 21–40\%$), 2+ ($A = 0.20–0.39/T = 41–60\%$), and 1+ ($A < 0.20/T > 60\%$).[2,27] Categories 6+ and 5+ included those high producers of biofilm isolates and categories 2+ and 1+ included poor or non-producers of biofilm. Categories were compared using the Chi-squared test with Yates' correction when necessary.

Table 1
Metabolic activity (XTT) of biofilms produced by *Candida albicans* and *Candida dubliniensis* clinical isolates at 48 h according to their clinical origin.

Species	Biofilm production (XTT metabolic production)	
	Blood	Oral
C. albicans	1.007 ± 0.401	0.900 ± 0.366
C. dubliniensis	0.522 ± 0.198	0.640 ± 0.163
Reference strains		
C. albicans NCPF 3153	0.550 ± 0.024	
C. dubliniensis NCPF 3940	0.634 ± 0.069	
C. albicans CA-2	0.112 ± 0.008	

Differences between the species were considered to be statistically significant at $P < 0.05$.

Results

All tested isolates of *C. albicans* and *C. dubliniensis* produced biofilm on polystyrene (Tables 1 and 2 and Fig. 1). However, a great variability in biofilm production was observed in both species. The biofilm metabolic activities (A492 nm) of *C. albicans* isolates at 24 h ranged between 0.291 and 1.506 (mean 0.869 ± 0.352) and those of *C. dubliniensis* isolates ranged between 0.438 and 1.145 (mean 0.688 ± 0.199) ($P = 0.03$) (Fig. 1A). *C. albicans* blood isolates showed a higher metabolic activity in 24 h biofilms than in oral isolates (0.955 ± 0.379 vs. 0.805 ± 0.328, respectively, $P = 0.28$). A similar pattern was observed for *C. dubliniensis* (0.763 ± 0.195 vs. 0.634 ± 0.192, respectively, $P = 0.17$).

Biofilm metabolic activities of *C. albicans* isolates at 48 h were higher than at 24 h (0.945 ± 0.378 vs. 0.869 ± 0.352, respectively, $P = 0.44$) with independence of the origin of these isolates (blood vs. oral isolates: 1.007 ± 0.401 vs. 0.900 ± 0.366, respectively, $P = 0.48$). However, at 48 h the biofilms of oral isolates of *C. dubliniensis* showed more metabolic activity (0.640 ± 0.163) than the biofilms of blood isolates (0.522 ± 0.198) ($P = 0.19$) (Table 2 and Fig. 1B). Most *C. dubliniensis* isolates belonged to genotype I, except isolates 00-133 and 00-135, which were from the genotype II (Fig. 1). There were no statistically significant differences in biofilm production between both genotypes.

C. albicans included a non-statistically significant higher number of isolates classified as high producers of biofilm than *C. dubliniensis* ($P = 0.86$) at 48 h. Ten out of 28 *C. albicans* isolates (35.7%) were classified as high producers of biofilm at 24 h. Of these, 5 were from blood (41.7% of the blood isolates) and 5 from the oral cavity (31.3% of the oral isolates) ($P = 0.86$). This category of high producers of biofilm only grouped 2 out of 19 *C. dubliniensis* isolates (10.5%) at 24 h, but this difference between isolates of both species was not statistically significant ($P = 0.109$). However, at 48 h, 10 out of 28 *C. albicans* isolates (35.7%) and 0 out of 19 *C. dubliniensis* isolates were classified as high producers of biofilm ($P = 0.01$). At this incubation time, 5 out of 12 (41.7%) blood isolates of *C. albicans* were high producers of biofilm in comparison to 5 out of 16 oral isolates (31.3%) ($P = 0.86$).

Discussion

The phenomenon of biofilm formation by microbes on inert surfaces has been extensively studied in bacteria and to a lesser extent in fungi, and there appears to be a direct relationship between the capability of the organisms to form a biofilm and their pathogenicity. However, few studies have compared biofilm production among *C. albicans* bloodstream and oral isolates[28] and much less, between oral and blood isolates of *C. dubliniensis*. The current results confirm that most *C. albicans* and *C. dubliniensis* oral and blood isolates develop biofilms on polystyrene plates. We used the colorimetric method based on the XTT reduction in the current study because it correlates well with other quantitative methods, such as ATP or CFU assays used in cross-comparison of species variation in biofilm production.[29] The XTT method is more versatile and less-time consuming, and is also particularly suited for assays using microtitre plates that allow the screening a large number of isolates.

Shin et al.[28] observed that a very low percentage of isolates of *C. albicans* produced biofilm, regardless of the invasive (bloodstream) or non-invasive origin of the isolates (8% vs. 7%). Conversely, the biofilm production was high when isolates from other species were tested, and those from blood and deep tissues were statistically significant higher producers than those isolates from other clinical specimens. Kumar and Menon,[30] using the same medium and method for biofilm production, reported that 11 out of 18 *C. albicans* isolates (61%) were biofilm producers. Three out of 7 bloodstream isolates (43%) produced biofilm. Non-*C. albicans* isolates were higher producers of biofilm than those of *C. albicans*. These authors included 5 reference strains of *C. dubliniensis*, observing that all of them produced biofilm. In contrast, other authors have reported that isolates of *C. albicans* produced statistically significantly more biofilm than other *Candida* species.[27,29,31–34] All isolates in our study showed a variable capability to produce biofilm, being distributed into 3 categories (from high to low producers of biofilm) considering their metabolic activities, and those isolated from the bloodstream were non-statistically significantly higher producers of biofilm than oral ones.

These discrepancies could be associated with differences in the methodology used, to the potential variability associated to the geographic origin of the isolates, or to other isolate-associated factors. Shin et al.[28] and Kumar and Menon[30] used a Sabouraud dextrose broth, and a RPMI-based broth was used in our comparison. Sabouraud dextrose broth with a high content of glucose (8%) has been claimed to simulate the hyperglycaemic milieu found in those patients receiving total parenteral nutrition.[28,30] However, RPMI-based broth is a better promoter of filamentation than Sabouraud

Table 2
Distribution of clinical isolates of *C. albicans* and *C. dubliniensis* on the basis of their capability for producing biofilm at 48 h.

Category	Biofilm	Species (no. of isolates included and percentage)				
		C. albicans		*C. dubliniensis*		Total
		Blood 48 h	Oral 48 h	Blood 48 h	Oral 48 h	48 h
High producers of biofilm	6+	2 (16.7%)	1 (6.3%)	0 (0%)	0 (0%)	3 (6.4%)
	5+	3 (25%)	4 (25%)	0 (0%)	0 (0%)	7 (14.9%)
Producers of biofilm	4+	4 (33.3%)	6 (37.5%)	1 (12.5%)	2 (18.2%)	13 (27.7%)
	3+	3 (25%)	5 (31.3%)	5 (62.5%)	9 (81.8%)	22 (46.8%)
Low producers and non-producers of biofilm	2+	0 (0%)	0 (0%)	2 (25%)	0 (0%)	2 (4.3%)
	1+	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total		12 (100%)	16 (100%)	8 (100%)	11 (100%)	47 (100%)

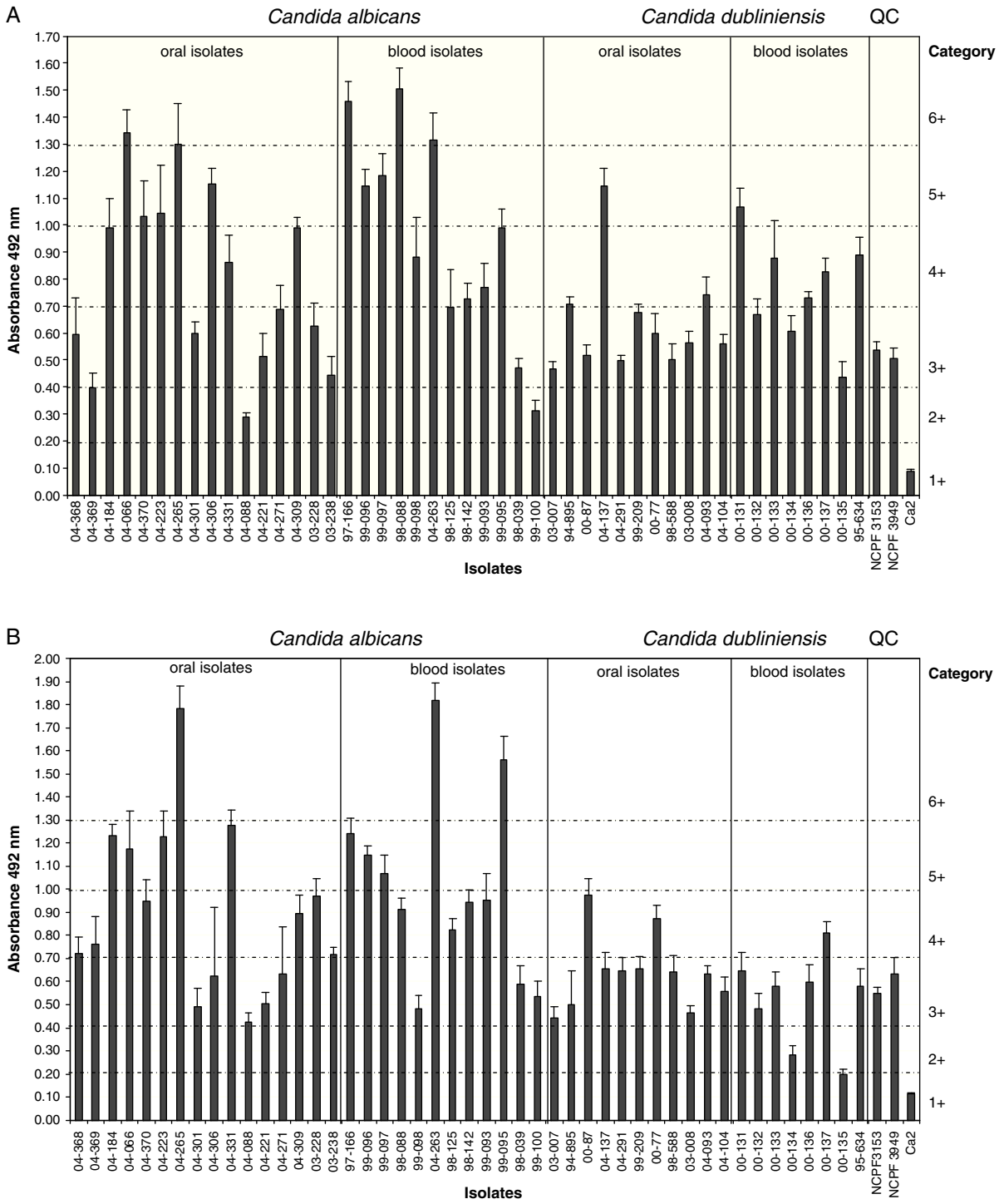

Fig. 1. Distribution of *Candida albicans* and *Candida dubliniensis* isolates on the basis of their capability for producing biofilms at 37 °C for 24 h (A) and 48 h (B).

dextrose broth,[35] a fact that is essential for the first step (adhesion) of biofilm production and can be influenced by different cultural and host factors.[36–38] This influence of the RPMI in germ tube and hypha development could explain the production of biofilm by all the *C. albicans* and *C. dubliniensis* isolates tested in the current study.

The geographical origin of isolates in the different studies can influence the biofilm production capability, as has been observed for other phenotypic traits of *Candida* isolates, such as virulence factors or *in vitro* antifungal susceptibilities.[29,39] This can be another potential explanation of the differences encountered, as Shin et al.[28] studied isolates from Korea, and Kumar and Menon[30] from India, while in the current study all *C. albicans* isolates were from Spain. Li et al.,[40] studying Canadian isolates of *C. albicans*, reported that natural clones and clonal lineages of *C. albicans* exhibited extensive quantitative variations in biofilm formation. Something similar was described by Borecka-Melkusova and Bujdakova[41] in *C. albicans* and *C. dubliniensis* isolates from Slovakia. Thein et al.[29] also reported that growth and virulence of the different species of *Candida* were highly dependent on the isolates chosen for each study, hence it is important to use multiple clinical isolates and strains for the same species to draw firm conclusions in this regard.

There was a high variability in the biofilm production among *C. albicans* isolates when the interspecies variation of *C. albicans* and *C. dubliniensis* isolates was studied. This variability was lower among *C. dubliniensis* isolates. Kuhn et al.[42] observed that XTT results can vary in accordance with the different sensitivity of *C. albicans* and *Candida parapsilosis* to tetrazolium salts. *C. dubliniensis* is more closely related to *C. albicans* than *Candida parapsilosis* and probably has a similar sensitivity to XTT than *C. albicans*. Nevertheless, we found that both *Candida* species, *C. albicans* and *C. dubliniensis*, exhibited good biofilm forming capability on polystyrene surfaces under aerobic conditions. These findings are in agreement with those of Ramage et al.[24] who also reported that *C. dubliniensis* exhibited good biofilm growth on the surface of polystyrene plates.

One of the key morphogenesis factors for the phase transition in these dimorphic fungi is the nature of the environment, as the hypha growth is promoted by low oxygen and, nutrient starvation, as occurs in this aerobic and static conditions assay.[29] Such a phase transition is dependent upon strain as well as species characteristics, as shown here by the CA-2 hypha-deficient strain of *C. albicans*, which always exists in the yeast phase, irrespective of its environmental milieu. Both species formed heterogeneous biofilms with strain variations in their morphology and XTT metabolic activity, as has been described previously by Henriques et al.[32]

In conclusion, there are important differences in biofilm production by *C. albicans* and *C. dubliniensis* isolates. These differences should be taken into account when the anti-biofilm activity of antifungal agents or other virulence factors are tested *in vitro*. This capability for biofilm development may enable *C. albicans* and *C. dubliniensis* to maintain their oral ecological niches as commensal microorganisms and can be a major virulence factor during invasive candidiasis with important clinical repercussions.

Conflict of interests

The authors have no conflict of interest to declare.

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