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RESEARCH ARTICLE

Real-time Assessment of Interspecies *Candida* Biofilm Formation

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Abstract:

Background:

Candida infections are responsible for increased morbidity and mortality in immunocompromised patients, with *Candida* having the ability to form highly structured biofilms that protect them from the external environment and the action of antifungals. Few studies have reported on real-time interactions between *Candida* species.

Objective:

This study aimed to investigate the interspecies biofilm changes of oral *Candida* species using the xCELLigence system and the CV staining technique.

Methods:

This study demonstrated the mixed biofilm interactions of *Candida* species using an impedance-based biofilm monitoring system and crystal violet staining.

Results:

When using the xCELLigence system, the maximum cell index increased in most mixed biofilms, except for the *C. glabrata*/*C. parapsilosis* and *C. albicans* combinations. Bulk biofilm formation measured by CV staining was the highest in *C. albicans* and *C. tropicalis* combinations and the lowest in the *C. glabrata*/*C. parapsilosis* combination. Pseudohyphae were observed in *C. albicans* and *C. glabrata* in combination with *C. tropicalis* or *C. parapsilosis*.

Conclusion:

This study is the first to report on the real-time interactions of *Candida* species using the xCELLigence system and suggests that the presence of specific species influences the biofilm formation of commonly isolated *Candida* species.

Keywords: *Candida*, Biofilms, Cell adhesion, CV staining, xCELLigence system, Species interactions.

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1. INTRODUCTION

Infections by commensal yeasts of the *Candida* genus range from superficial mucosal infections to disseminated infections. Candidiasis is the most frequent of the opportunistic infections, occurring in patients with underlying disease or those on broad-spectrum antibiotics or immunosuppressive therapy. Oral candidiasis usually presents with yellowish-white plaques on the oral mucosa (oral thrush), while the erythematous form of candidiasis, associated with denture wear or poor denture hygiene [1], consists of bright red spots on the tongue or palate. *Candida* is also involved in the development of angular cheilitis and has been associated with oral cancer [2] and dental caries [3]. It is present in virtually all HIV-positive patients with severe immunosuppression, having the ability to spread to other body sites and resulting in increased patient morbidity and mortality.

Topical polyene antifungals such as amphotericin B and nystatin or imidazole drugs such as miconazole and clotrimazole are used to manage localised oral *Candida* infections [4]. However, these infections often recur. In

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denture-associated erythematous candidiasis, the most common form of candidiasis, the localised reddening of the mucosa beneath the fitting surface of dentures can be treated with either antifungals, denture cleansing agents, natural extracts or denture base resin coatings, which may reduce the attachment of *Candida albicans* (*C. albicans*) to dentures [5].

Cases requiring systemic antifungal therapy are treated with either triazoles, which have fewer side effects than other drug classes [6], the more recently developed echinocandins, which are used to treat infections by the inherently azole-resistant species *Candida glabrata* (*C. glabrata*) and *Candida krusei* (*C. krusei*) [7, 8] or the fluorinated pyrimidine 5-flucytosine, which has considerable side effects and is often ineffective when used on its own [9]. Due to its low cost, fluconazole is routinely administered for candidiasis in healthcare facilities on the African continent when topical antifungal treatment is ineffective and for the treatment of oropharyngeal or oesophageal candidiasis [4]. However, the resistance to this drug is increasing [6]. Drug-resistant, non-albicans species have emerged and are increasing due to severe patient immunosuppression or the indiscriminate use of broad-spectrum antimicrobials [10].

Distinct *Candida* species express different virulence factors that allow them to attach to the host tissues, evade the action of innate immune responses and survive exposure to antifungals [11, 12]. Furthermore, *Candida* species can form polymicrobial biofilms, including those composed of multiple combinations of *Candida* species [13]. Biofilms provide microbial communities with metabolic cooperation and protection from the external environment and antimicrobial drugs [14], with different *Candida* species demonstrating differences in biofilm formation, morphology, characteristics of the extracellular matrix (ECM) and the ability to confer antifungal resistance [15, 16].

Biofilm formation involves the processes of adhesion, morphologic modifications, increases in cell number and the production of the ECM, which facilitates the adhesion between cells in the biofilm and the environment or substrate they colonise [17]. *C. albicans* is known to have a higher biofilm-forming ability than non-albicans species, and its biofilms were shown to be affected when grown with *Candida rugosa* [18], *Candida tropicalis* (*C. tropicalis*) and *C. krusei* [19]. Most previously published studies on *Candida* biofilms employed either the 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay, which measures metabolic cell activity [20] or the crystal violet (CV) staining technique, an assay that measures the uptake of crystal violet by already formed biofilms [21]. However, the XTT reduction assay has been criticised for having a low detection limit and being difficult to apply in polymicrobial biofilms (due to different metabolic rates of organisms), while the CV staining technique can lead to an overestimation or underestimation of biofilm biomass [22]. These methodologies are also not able to measure different biofilm formation parameters over time.

The xCELLigence real-time cell analyser is an impedance-based monitoring system that allows for real-time monitoring of changes in cell number, size, adhesion and extra-cellular polymeric substances (EPS) biofilm formation and continuous and automated data analysis. This system, which has been used to assess the biofilm formation abilities of *C. albicans* [23, 24], relies on microtiter plates with gold microelectrodes through which impedance signals (expressed as cell index, CI) are measured and assesses the cell adhesion /EPS formation dynamics over time. When biofilm development occurs, the CI values initially increase as the biofilm adheres and proliferates on the bottom of the E-plate wells, with a peak CI being consistent with the start of the biofilm maturation stage and a decrease taking place when the biofilm initiates the detachment phase [25]. A declining slope after the peak CI value has been suggested as a measure of biofilm formation [26]. As the polysaccharides that hold the biofilm together are broken down by enzymes, releasing the cells from the biofilm matrix, the electrical signals are also further affected by extracellular components and physical appendages of microorganisms, which are consistent with the death phase [27]. There is a paucity of data related to the real-time mixed *Candida* species biofilm dynamics using this method.

This pilot study investigates the interspecies biofilm changes of oral *Candida* species using the xCELLigence system and the CV staining technique.

2. MATERIALS AND METHODS

2.1. Organisms used in this Study

Candida type strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and National Collection of Pathogenic Fungi (NCPF, Public Health Culture Collections, England) and included *Candida albicans* ATCC 90028, *Candida dubliniensis* NCPF 3949a, *Candida glabrata* ATCC 26512, *Candida tropicalis* ATCC 950, *Candida krusei* ATCC 2159 and *Candida parapsilosis* ATCC 22019. These six isolates were revived by growth in 10 ml Sabouraud dextrose broth (Cat. no. CMO147, Oxoid, UK), followed by incubation at 37 °C for 3–5 days. Purity of growth was confirmed by microscopy and subculture of single colonies previously grown on Sabouraud dextrose agar (Cat. no. 84088, Sigma-Aldrich, USA) and incubated aerobically at 37 °C for 24-48 h. Species differentiation was confirmed by growth on Fluka chromogenic *Candida* identification agar (Cat. no. 94382; Sigma-Aldrich, St. Louis, MI, USA) and Oxoid chromogenic *Candida* agar (Cat. no. CM1002A; Oxoid, Hampshire, UK) at 30 °C for 24–72 h.

2.2. Real-time Monitoring of Biofilm Formation

Prior to conducting this study, preliminary toxicity tests were performed by growing the organisms in the presence of other species' sterile supernatants, as previously described [24], to assess whether any organisms induced toxicity against the others. The adhesion changes of the individual isolates were tested in yeast peptone dextrose (YPD) broth (Cat. no. Y1375, Sigma-Aldrich, USA), a medium known to promote *Candida* adherence and biofilm formation [24, 28, 29] with 100 µl of a 1:20 dilution of a 0.5 McFarland standard suspension and 100 µl of YPD broth added to the wells of an E-plate 16 (Cat. no. 05469830001, ACEA Biosciences, USA). When testing the mixed growth of two species, *C. albicans*, *C.*

dubliniensis and *C. glabrata* were grown in the presence of the less commonly isolated oral species *C. tropicalis*, *C. krusei* and *C. parapsilosis*. Fifty microliters (50 µl) of a 1:20 dilution of a 0.5 McFarland standard suspension of each organism was added to an individual E-plate 16 well, resulting in a total volume of 100 µl cell suspension and a further 100 µl of YPD broth added to the well. Sterile distilled water was placed in the surrounding evaporation-control troughs, and the plate was inserted into the RTCA plate analyser (Cat. no. 05469759001, ACEA Biosciences, USA), previously placed in a 37°C incubator. An experimental procedure was logged on the RTCA software package (Cat. no. 05454433001, ACEA Biosciences, USA), with impedance readings set to take place at 15-minute intervals for 48 h. The real-time CI values for the full duration of the experiments were then plotted in individual graphs. Experiments were duplicated, and sterility controls were included in every tested plate.

2.3. Bulk Biofilm Morphology

Bulk biofilm morphology was observed by growing the organisms on 12-well cell culture plates (Cat. no. 3513, Corning, USA). Two millilitres (2 ml) of 0.5 McFarland standard Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Cat. no. R6504, Sigma-Aldrich, USA) suspensions of the *Candida* isolates were dispensed into 12-well plates, followed by shaking at 37°C for 90 min at 75 rpm to allow the cells to adhere to the bottom of the wells. The medium was subsequently aspirated, and the wells were gently washed with 2 ml phosphate buffered saline (PBS) pH 7.4 (Cat. no. P3813, Sigma-Aldrich, USA), followed by the addition of fresh RPMI-1640 medium and shaking at 37°C for 24 and 48 h at 75 rpm. For the 48 h growth, the medium was replenished after 24 h. Controls with the medium but no cultures were included. The medium was then aspirated, and the wells were washed with 2 ml PBS to remove unattached cells. Fluorescence microscopy (FM) was used to observe both the monospecies and mixed-species biofilm morphology. A volume of 100 µl 0.05% (vol/vol) calcofluor white stain (Cat. no. 18909, Sigma-Aldrich, USA) that binds to chitin in fungal cell walls was added to the wells in a dark room together with 50 µl of a 10% potassium hydroxide solution, for better observation of fungal structures. The formed biofilms were observed under ultraviolet (UV) light at 40 X magnification using an Eclipse Ts2-FL epi-fluorescence inverted microscope (Nikon Inc., Japan) with an excitation of 300 to 440 nm, with pictures taken at 6-20 ms exposure.

2.4. CV Staining

For the CV staining assay, individual colonies of freshly grown *Candida* cultures were transferred to sterile test tubes containing filter-sterilised RPMI-1640 medium and MOPS (N-Morpholino-propanesulfonic acid) (Cat. no. M1254, Sigma-Aldrich, USA) 0.165 M, but without sodium bicarbonate (pH 7.0). After adjusting the inoculum to a 0.5 McFarland standard suspension, suspensions of individual cultures were added to a 96-well microtiter plate (200 µl for individual species and 100 µl for each species for mixed growth). Experiments for each condition were repeated four times and included sterility and negative controls in each tested plate.

The plates were sealed using sealing membranes (Cat. no. P2SEA002Z, Lasec, South Africa) and shaken at 37 °C for 90 min at 75 rpm in a shaking incubator to allow the cells to adhere to the well surfaces. The medium was subsequently aspirated, and the wells were washed with 150 µl PBS to remove loosely adherent cells. After PBS aspiration, 150 µl of fresh RPMI-1640 medium was added to the wells, followed by incubation at 37°C for 66 h at 75 rpm. The medium was aspirated daily, and 150 µl fresh RPMI-1640 medium was added to the wells. The medium was then aspirated, and the wells were washed twice with 200 µl PBS and air-dried for 45 min, followed by staining with 110 µl of 0.4% aqueous CV solution for 45 min, washed four times with 200 µl sterile distilled water and de-stained for 45 min in 200 µl of a 95% ethanol solution, to provide a decolourisation solution which serves as a proxy for the number of cells within the biofilm [30, 31]. A volume of 100 µl of the solution was then transferred from each well to a new plate, and the absorbance was read at OD_{595} using a FLUOstar Omega spectrophotometer plate reader (BMG Lab-Tech, Germany). Absorbance values for the controls were subtracted from test well values to minimise background interference. The mean absorbance values of all replicate tests were used to compare the biofilm formation results.

3. RESULTS

3.1. Real-time Biofilm Formation

No toxicity-associated growth reduction was observed with any combination of species and supernatants. All tested species formed biofilms after 7-10 h except for *Candida parapsilosis* (*C. parapsilosis*), which had negative CI values when grown on its own (Fig. **1**).

Peak CI values occurred between 15-17 h for individual species, except for *C. glabrata* (11 h). *C. albicans* had the highest CI values of all tested species, followed by *C. glabrata*. The highly virulent *C. tropicalis* and the inherently azole-resistant *C. krusei* also resulted in positive CI values (indicative of adhesion and biofilm formation) when grown independently. The adhesion of mixed species over time was higher than in monocultures, except for the *C. albicans* and *C. parapsilosis* combination (Fig. **1**). Although the peak CI values for most combined *Candida* species were higher than those of individual species, the maximum CI for *C. albicans* was reduced when in the presence of any of the three species tested, while *C. glabrata* had a noticeably lower maximum CI when combined with *C. parapsilosis* (Table **1**).

Table 1. Mean maximum Cell Index (CI) values for individual and combined *Candida* species, measured using impedance.

Mean Individual and Combined Species Maximum CI-Values			
	Ct (0.67)	Ck (0.85)	Cp (-0.17)
Ca (1.65)	1.3	1.35	1.4
Cd (0.74)	1.17	1.13	0.94
Cg (0.99)	1.35	0.98	0.82

Note: Ca: *C. albicans*; Cd: *C. dubliniensis*; Cg: *C. glabrata*; Ct: *C. tropicalis*; Ck: *C. krusei*; Cp: *C. parapsilosis*.

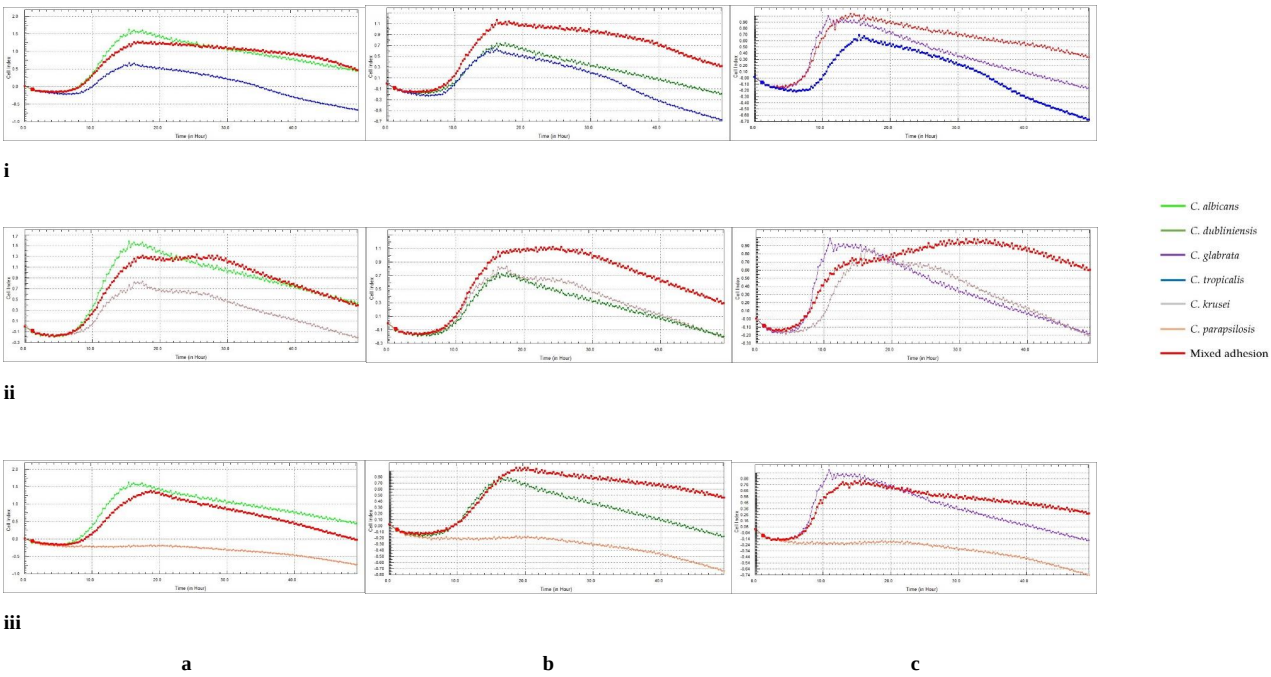

Fig. (1). xCELLigence system CI[1] variations of (**a**) *C. albicans* (light green), (**b**) *C. dubliniensis* (dark green) and (**c**) *C. glabrata* (purple) with (**i**) *C. tropicalis* (blue), (**ii**) *C. krusei* (light grey) and (**iii**) *C. parapsilosis* (coral) biofilm formation, with the mixed adhesion shown in red. The CI (y-axis) is plotted against the time in hours (x-axis). The adhesion of mixed biofilms over time was higher than in monocultures, except for the *C. albicans* and *C. parapsilosis* combination. Peak CI values for species combinations were higher than monospecies, with the exception of *C. albicans* combinations and the *C. glabrata* and *C. parapsilosis* combinations.

[1] CI, cell index

3.2. Bulk Biofilm Morphology Evaluation using FM

When growing the monospecies *Candida* biofilms, increased biofilm density between 24 h and 48 h was most notable in *C. albicans*, *Candida dubliniensis* (*C. dubliniensis*), *C. glabrata* and *C. tropicalis*, which showed a marked increase in biofilm mass after 48 h (shown as confluent growth in the well surface and increased fluorescence) (Fig. **2**). When grown independently, *Candida parapsilosis* failed to demonstrate a noticeable increase in biofilm mass between 24 and 48 h.

When observing the mixed species biofilms, increased cell density indicated the interactions between *C. albicans* with *C. tropicalis* and *C. parapsilosis*. *C. parapsilosis* combinations with *C. dubliniensis* and *C. glabrata* also demonstrated increased cell density. The *C. tropicalis* combinations with *C. glabrata* and *C. dubliniensis* also resulted in a notable increase in cell density.

Candida albicans and *C. parapsilosis* appeared to form germ tubes and filamentous structures (pseudohyphae and hyphae) as part of their monospecies biofilms, while extensive filamentous forms were prominent in the *C. tropicalis* monoculture biofilm. In the case of the mixed biofilms, pseudohyphae were extensive in the *C. albicans* combinations with *C. tropicalis* and *C. parapsilosis*, as well as the *C. glabrata* combinations with the same two species (Fig. **2**). A transparent mass surrounding large clusters of cells was also observed, indicating EPS formation.

3.3. Bulk Biofilm Quantification using CV Staining

The 66-hour bulk biofilm production absorbance values (A_{595}) in individual wells ranged between 0.89 and 3.33. When looking at the bulk biofilm production of individual species, *C. albicans* formed the greatest mass of biofilm, followed by *C. tropicalis* and the other species (*C. albicans* > *C. tropicalis* > *C. krusei* > *C. parapsilosis* > *C. dubliniensis* > *C. glabrata*). The biofilm production of combined species was the highest for *C. albicans*/*C. tropicalis* followed by the mixed biofilm combinations of these and other species (*Ca*/*Ct* > *Cg*/*Ct* > *Cd*/*Ct* > *Ca*/*Cp* > *Ca*/*Ck* > *Cg*/*Ck* > *Cd*/*Ck* > *Cd*/*Cp* > *Cg*/*Cp*).

4. DISCUSSION

When using both the novel xCELLigence system and the conventional CV staining method, *C. albicans* demonstrated the highest absorbance/impedance values when compared to the other species studied, supporting previous studies which have described it as the most notorious biofilm former of all pathogenic yeasts [14, 32]. Most xCELLigence mixed growth curves appeared to follow similarly (albeit increased) adhesion, maturation and detachment phase curves, an interpretation of biofilm formation first described by Gutiérrez *et al.* [25].

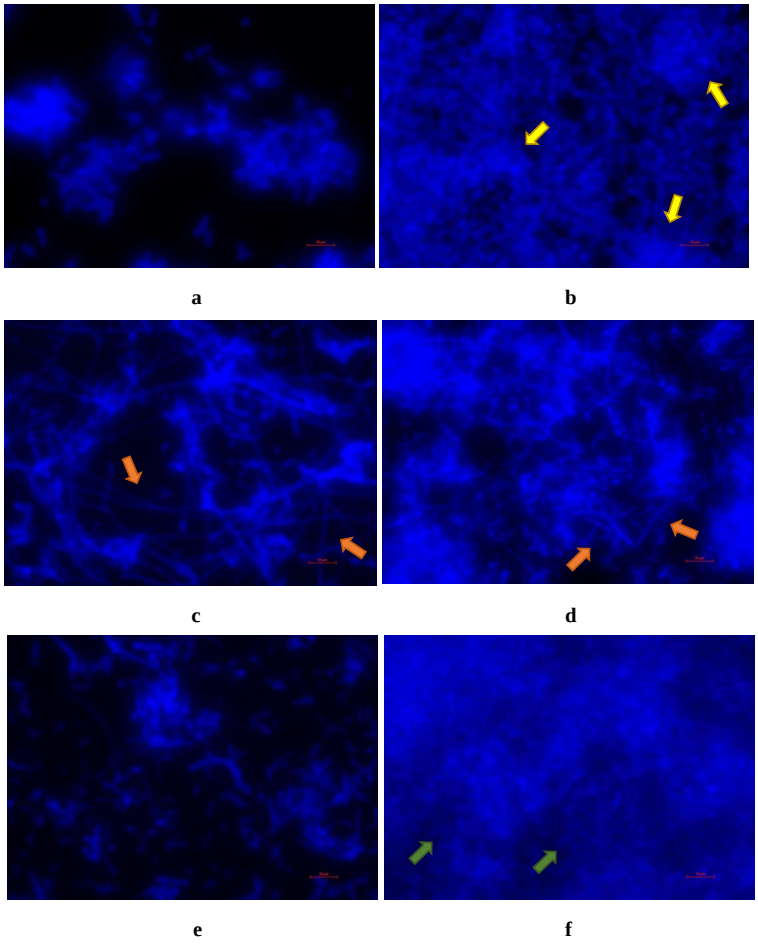

Fig. (2). Early (24 h) and mature (48 h) bulk biofilm observation using fluorescence microscopy after organisms were grown in 12-well cell culture plates containing RPMI 1640 medium with L-glutamine and stained with calcofluor white stain and a 10% potassium hydroxide solution. The pictures show increased biofilm density (in **a** and **b**, pointed by yellow arrows, where a *C. dubliniensis* monoculture is shown), the presence of filamentous structures (**c** and **d**, pointed by orange arrows, where combined growth of *C. glabrata* and *C. tropicalis* is shown) and EPS formation (**e** and **f**, pointed by green arrows, where combined growth of *C. dubliniensis* and *C. tropicalis* is shown). An increase in biofilm mass is shown as confluent growth in the well surface and increased fluorescence and was most noticeable in *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. tropicalis* monocultures and in the interactions of *C. albicans* with *C. tropicalis* and *C. parapsilosis*, *C. dubliniensis* interactions with *C. tropicalis* and *C. parapsilosis* and *C. glabrata* interactions with *C. tropicalis* and *C. parapsilosis*. Filamentous structures were observed in *C. albicans*, *C. parapsilosis* and *C. tropicalis* monospecies biofilms and when *C. albicans* and *C. glabrata* were grown in combination with either *C. tropicalis* or *C. parapsilosis*, while EPS formation could be seen as a transparent mass surrounding clusters of cells. Magnification = 40 X.

C. parapsilosis is less likely to produce biofilms, and when it does, the biofilm tends to be thinner and less structured than *C. albicans* [33], probably due to *C. parapsilosis* biofilms having less extracellular material compared to *C. albicans*, *C. glabrata* and *C. tropicalis* biofilms [33]. When using the CV method in our study, *C. parapsilosis* formed less bulk biofilm than *C. albicans*, *C. tropicalis* or *C. krusei* when grown on their own, and although its presence resulted in biofilm formation when combined with *C. albicans*, it had much lower biofilm abilities when combined with the other species. Although *C. parapsilosis* did not form biofilms when grown using the xCELLigence system, it increased CI values over time when combined with *C. dubliniensis* and *C. glabrata*. These results could be explained by the fact that the xCELLigence system, which provides continuous monitoring of biofilm formation and automated data analysis, takes into consideration various factors such as changes in cell number and size, adhesion and EPS formation (as opposed to only the bulk biofilm formation measured by CV staining) and that in biofilms, because matrix assembly is coordinated extracellularly, synergistic activity between species may restore matrix structure and function when species lacking a particular carbohydrate can be complemented by neighbouring cells lacking a different carbohydrate [34].

Although an increase in biofilm mass over time was not observed for *C. parapsilosis*, fluorescence microscopy allowed for the observation of increased cell density when this organism was grown with the other species, demonstrating interspecies synergism when *C. parapsilosis* was present. This technique allowed for the observation of cell density and filamentation. Our results concur with previous studies showing extensive filamentation in *C. albicans* biofilm formations with *C. tropicalis* and *C. parapsilosis* [35, 36] and *C. glabrata* [37, 38]. This switch to pseudohyphal and hyphal morphologies has been associated with increased invasion, penetration and growth in between host epithelial cells [39],

endothelial cell damage [40] and the destruction of immune cells following phagocytosis [41]. It can therefore be inferred that the extensive pseudohyphae observed in the aforementioned species combinations increase *Candida* virulence in affected hosts, leading to increased patient morbidity.

A limitation of this study is that we did not include biofilm-defectant mutants of *Candida* . Homozygous insertions in NUP85, MDS3, KEM1 and SUV3 were reported to block biofilm development in *C. albicans* in the early stage of biofilm formation [42] and suggested that *Candida* mutants are defective in the production of hyphae, which are thought to anchor the biofilm structure. This study provides the basis for further studies to explore the effect of mutant strains on mixed biofilm formation using this system.

CONCLUSION

Since adhesion is the first step towards the colonisation and invasion of host cells during the infectious process, the ability of the xCELLigence system to (i) monitor, in real-time, the adhesion and formation stages of *Candida* biofilms, (ii) assess other biofilm formation parameters and (iii) reveal the distinct phases of biofilm formation [26] could provide a distinct advantage over other biofilm assessment techniques (such as the CV staining method, which only quantifies the bulk biofilm), making it the method of choice for the assessment of interspecies biofilm formation.

The results of this study show that *C. albicans*, *C. dubliniensis* and *C. glabrata* enhance the biofilm formation of *C. tropicalis*, *C. krusei* and *C. parapsilosis*, all of which have different antifungal susceptibility profiles. This is important for the consideration and application of antifungal drugs for the treatment of resistant *Candida* biofilms. The authors are satisfied that the study's objective was achieved while also recognising the need for further studies to address the nature of the adhesins involved and the morphological changes that may occur.

LIST OF ABBREVIATIONS

CV	=	Crystal Violet
EPS	=	Extra-cellular Polymeric Substances
YPD	=	Yeast Peptone Dextrose

AUTHORS' CONTRIBUTION

Conceptualization, C.A.; methodology, C.A. and P.A.; investigation, P.A. and K.B.; writing—original draft preparation, P.A.; writing—review and editing, C.A.; visualization, P.A. and K.B.; supervision, C.A. and P.A.; funding acquisition, C.A. and P.A. All authors gave their final approval and agreed to be accountable for all aspects of the work.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

AVAILABILITY OF DATA AND MATERIALS

All data is included in the manuscript, and there is no additional data to deposit.

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