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Interactions between *Candida albicans* and *Candida glabrata* in biofilms: Influence of the strain type, culture medium and glucose supplementation

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Summary

The relationship among *Candida* species may be influenced by several factors. Thus, this study evaluated the interactions between *Candida albicans* and *Candida glabrata* in biofilms, varying the strain type, culture medium and glucose supplementation. Biofilms were formed for 48 hours in Sabouraud dextrose broth (SDB) or RPMI 1640, supplemented with 0%, 1% or 5% glucose. Each strain of *C. albicans* was combined with two strains of *C. glabrata*, generating four biofilm associations, which were quantified by colony-forming units (CFUs), total biomass and metabolic activity. Data were analysed by ANOVA and Tukey's HSD test ($\alpha = 0.05$). For CFUs, all associations were classified as indifferent for biofilms formed in RPMI 1640, while for SDB the interactions were antagonistic for *C. albicans* and indifferent for *C. glabrata*. The association of reference strains resulted in a dual-species biofilm with biomass significantly higher than that observed for each single biofilm developed in SDB. The metabolic activity of dual-species biofilms did not significantly differ from that found for single ones, except for co-culture of the reference strains. Glucose supplementation and culture media had a significant influence on all parameters. In conclusion, the strain type, culture medium and glucose supplementation influenced the interactions between *C. albicans* and *C. glabrata*.

KEYWORDS
biofilms, *Candida albicans*, *Candida glabrata*, microbial interactions

1 | INTRODUCTION

Candida species are known to be opportunistic pathogens that may be found in the oral cavity, bloodstream, and gastrointestinal, genitourinary and respiratory tracts of the human body of healthy and immunocompromised individuals.[1] These species are labelled as commensals when the individual's immune response and interactions of *Candida* with other microbial species allow their persistence (usually in low numbers) without causing harm to the host.[2,3] Nevertheless, alterations in the dynamic equilibrium between the host and microbial colonisation (eg, caused by immunodeficiency) may favour the atypical growth of *Candida* species, making them pathogenic and leading to clinical manifestations of candidiasis.[3] In this sense, it was recently demonstrated a higher prevalence of *Candida* species in the oral cavity of diabetic patients compared to non-diabetics, being *Candida albicans* the most frequently isolated microorganism from the two groups of patients.[4] This species was also the most found in the oral cavity of individuals carrying the human immunodeficiency virus.[5]

Candida albicans has ability to adhere and form biofilms on different surfaces,[6] exhibits phenotypic variability and cellular polymorphism,[7]

as well as capacity to synthesise extracellular enzymes[8] and to produce toxins, which contribute to its virulence. Other *Candida* species are also regarded as clinically important opportunistic pathogens, such as *Candida glabrata*.[9] For a long time, this microorganism was considered a fungus present in the microbiota of healthy individuals, without great pathogenic potential.[9,10] At present, however, *C. glabrata* is known to be one of the most prevalent pathogens of the genus *Candida*[11] and, in cooperation with *C. albicans*, are often involved in cases of oral candidosis, like denture stomatitis.[12] *C. glabrata* has no ability to form hyphae, but it is aggressive and resistant to antifungal agents.[13] Its virulence factors include the secretion of hydrolytic enzymes (phospholipases and haemolysins) and the capacity to form biofilms on biotic (mucosa) and abiotic surfaces (catheters, silicone, latex, acrylic and polyurethane).[9,10]

In the oral cavity, these fungi are mostly found in polymicrobial biofilms, and their heterogeneity complicates the assessment of the individual contribution of each species to the pathogenesis of the oral diseases.[14] In one of the first reports on this issue, Kirkpatrick et al[15] observed a competitive relationship between *C. albicans* and *Candida dubliniensis*. Similarly, clinical strains of *Candida krusei* significantly inhibited the metabolic activity and growth of *C. albicans* in mixed biofilms.[16] On the other hand, Pereira-Cenci et al[17] did not find competitiveness between *C. albicans* and *C. glabrata* in biofilms developed on different dental materials. In addition to the above-mentioned interactions among different strains, dietary sugars are factors that interfere with the oral colonisation and biofilm formation by *Candida* species. In this context, fungal cells developed in medium containing glucose displayed higher biofilm formation than yeasts grown in galactose-containing medium.[18] Likewise, glucose promoted higher counts of yeasts in comparison to sucrose.[17]

Although some studies have investigated the associations among different *Candida* species in mixed biofilms, there are no reports about the influence of the type of strain, culture medium and glucose supplementation on the interactions between *C. albicans* and *C. glabrata*. Therefore, the objective of this study was to evaluate the interactions between these species in mixed biofilms formed in vitro, taking into account the above-mentioned variables.

2 | MATERIALS AND METHODS

2.1 | *Candida* strains and growth conditions

Two *Candida* strains from American Type Culture Collection (ATCC) were used in this study: *C. albicans* ATCC 10231 and *C. glabrata* ATCC 90030. In addition, two clinical oral isolates were tested: *C. albicans* 324LA/94 and *C. glabrata* D1, obtained from the culture collection of Cardiff Dental School (Cardiff, UK) and the Biofilm Group of the Centre of Biological Engineering, University of Minho (Braga, Portugal), respectively. All strains were subcultured aerobically on Sabouraud dextrose agar (SDA; Difco, Le Pont de Claix, France) plates during 24 hours at 37°C. Afterwards, a loopful of each *Candida* strain was inoculated in 10 mL of Sabouraud dextrose broth (SDB; Difco) and incubated at 37°C for 20-24 hours in an orbital shaker at 120 rev/min. Next, the *Candida* cells were harvested by centrifugation (6500 × *g*; 5 minutes) and the pellets, washed twice with phosphate-buffered saline (PBS; 0.1 mol L^{-1}, pH 7). The number of fungal cells was adjusted to 1×10^7 in SDB or Roswell Park Memorial Institute (RPMI; Sigma-Aldrich, St Louis, MO, USA) 1640, both supplemented with glucose (Sigma-Aldrich, CAS number 50-99-7) at 0%, 1% or 5%, using a Neubauer counting chamber.

2.2 | Single- and mixed-species biofilm formation

Candida biofilms were formed in the 96-well microtiter plates (Costar, Tewksbury, MA, USA)[19] containing 200 μL of the standardised cell suspension of each strain in single culture (1×10^7 cells/mL in SDB or RPMI 1640, supplemented with glucose at 0%, 1% or 5%). Regarding mixed biofilms, 100 μL of each inoculum (2×10^7 cells/mL for *C. albicans* + 2×10^7 cells/mL for *C. glabrata*) was added into each well. The microplates were then incubated at 37°C for 48 hours, and the culture media (supplemented with glucose at 0%, 1% or 5%) were renewed every 24 hours. After the biofilm formation period (48 hours), each culture medium was removed and the wells, washed once with PBS to eliminate non-adherent fungal cells.

2.3 | Biofilm quantification assays

2.3.1 | Quantification of cultivable cells

The resulting biofilms were washed and resuspended with 200 μL of PBS, scraped from the wells and vigorously vortexed (1 minute) for breaking the cell aggregates. Next, serial decimal dilutions were prepared in PBS and plated on SDA and CHROMagar *Candida* (Difco), respectively, for single- and dual-species biofilms. The agar plates were aerobically incubated at 37°C and the number of colony-forming units (CFUs) was recorded after 24-48 hours. CFU results were expressed as a function of the area of the wells ($\log_{10}$ CFU cm^{-2}).

2.3.2 | Biomass quantification

Total biomass of single- and dual-species biofilms was quantified by the crystal violet (CV) staining method.[19] Briefly, *Candida* biofilms were fixed with 200 μL of 99% methanol (Sigma-Aldrich, CAS number 67-56-1), which was removed after 15 minutes of contact. The microtiter plates containing the fixed biofilms were allowed to dry at room temperature, and 200 μL of 1% CV (Sigma-Aldrich, CAS number 548-62-9) were then pipetted into each well. After 5 minutes of incubation, the CV excesses were removed, the wells were washed with deionised water, and 200 μL of 33% acetic acid (Sigma-Aldrich, CAS number 64-19-7) was added to release the CV stain from all biofilms. The absorbance values were obtained at 570 nm and standardised per unit area of the wells (Absorb cm^{-2}). The blanks were considered as wells containing SDB or RPMI 1640 supplemented with 0%, 1% or 5% glucose, without fungal cells.

2.3.3 | Evaluation of the metabolic activity

The XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide; Sigma-Aldrich, CAS number 111072-31-2) reduction assay was employed to assess the metabolic activity of the biofilm cells.[20] For this, a solution (200 μL) consisting of 150 mg XTT L^{-1} and 10 mg of phenazine methosulphate L^{-1} (Sigma-Aldrich, CAS number 299-11-6) was pipetted into each well after the biofilm formation period (48 hours), and the microplates were incubated in a shaker (120 rev/min) at 37°C protected from the light. After 3 hours, 200 μL of the supernatant was transferred to new 96-well microplates and the absorbance, measured at 490 nm. All absorbance values were standardised per unit area of the wells (Absorb cm^{-2}), and the blanks were similarly processed without cell suspensions.

2.4 | Classification of the microbial association

For dual-species biofilms, each *C. albicans* strain was combined with two *C. glabrata* strains, yielding four microbial associations: *C. albicans* ATCC 10231+ *C. glabrata* ATCC 90030, *C. albicans* ATCC 10231+ *C. glabrata* D1, *C. albicans* 324LA/94+ *C. glabrata* ATCC 90030 and *C. albicans* 324LA/94 + *C. glabrata* D1. The microbial association was classified according to Miceli et al,[21] with modifications. When the association promoted significant decreases in the number of CFUs, compared with each strain alone, it was considered to be antagonistic. However, when the association displayed a significant increase in the number of CFUs, compared with each strain alone, it was defined as synergistic. If the results of quantification of cultivable cells of mixed-species biofilms were not significantly different from those obtained for each strain alone, the association was designated as indifferent. As the CV and XTT assays do not allow to differentiate the biomass and metabolic activity of each species individually in mixed biofilms, these methods were not used to classify the microbial associations, but instead as adjunct data, to assist in the interpretation of the results obtained.

2.5 | Biofilm structure

The structure of single- and mixed-species biofilms was qualitatively evaluated by scanning electron microscopy (SEM). For this, the biofilms were formed in 24-well plates, during 48 hours, according to the protocol described above. Next, biofilm samples were processed as described by Monteiro et al,[22] and analysed in the FEG-VP Supra 35 electron microscope (Carl Zeiss, Jena, Thuringen, Germany).

2.6 | Statistical analysis

All biofilm assays were performed in triplicate on three separate occasions. The types of strain, culture media (SDB or RPMI 1640) and glucose supplementation (0%, 1% or 5%) were considered as variation factors. Data were analysed by three-way ANOVA and Tukey's HSD

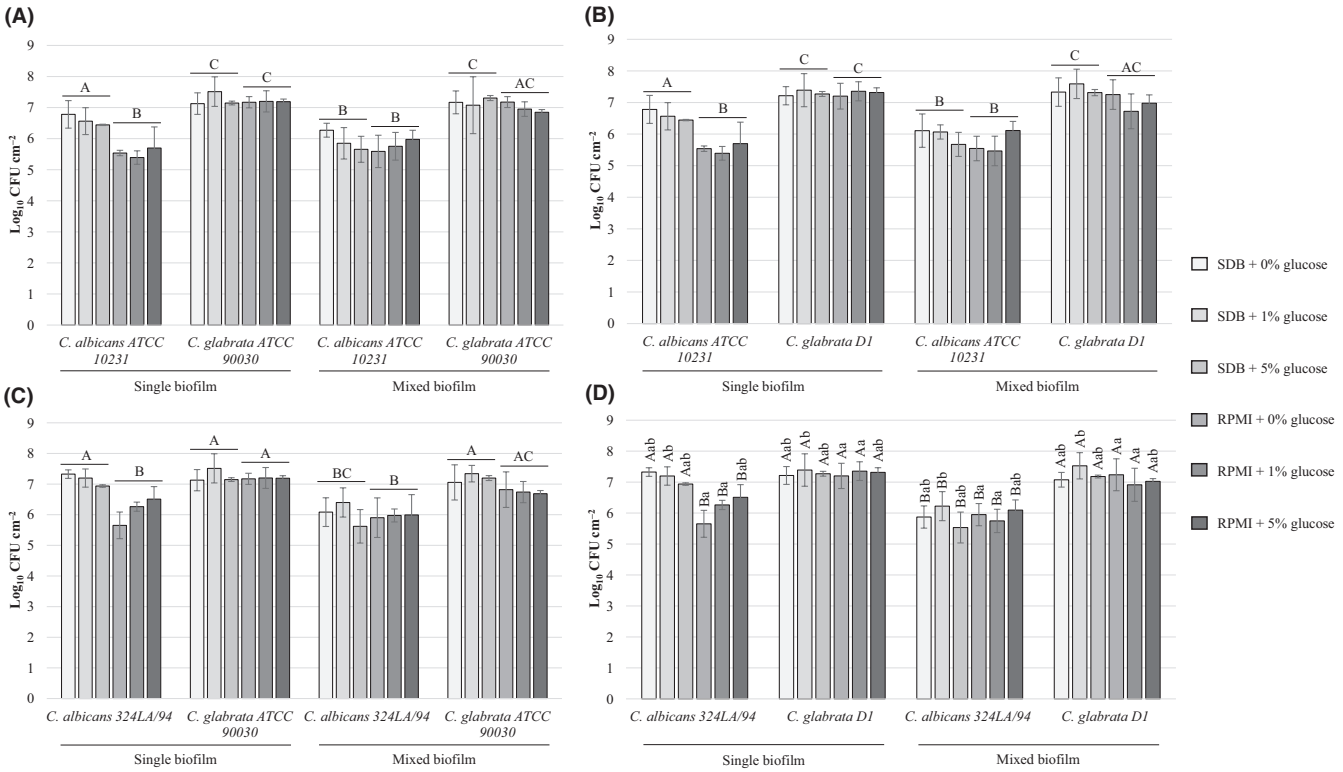

FIGURE 1 Logarithm of colony-forming units obtained from the interactions between *Candida albicans* and *Candida glabrata* in biofilms. For each microbial association, distinct letters represent significant differences among the biofilms for the interactions between strain and medium (upper case) and medium and glucose supplementation (lower case). Three-way ANOVA and Tukey's HSD post-hoc test, $P < .05$

post-hoc test (STATISTICA, version 10.0; TIBCO Software Inc., Palo Alto, CA, USA). The significance level was set at $P < .05$.

3 | RESULTS

3.1 | Quantification of cultivable cells

Regarding the number of CFUs, all microbial associations were classified as indifferent for biofilms formed in RPMI 1640 medium (Figure 1A-D), while for SDB medium the interactions were considered antagonistic for *C. albicans* and indifferent for *C. glabrata* (Figure 1A and B). Glucose supplementation only had a significant influence on the association of *C. albicans* 324LA/94 with *C. glabrata* D1, with a significant interaction between glucose supplementation and media. Overall, the number of CFUs was significantly lower for single- and mixed-species biofilms formed in RPMI 1640 supplemented with 1% glucose, compared with SDB medium supplemented with glucose at the same concentration (Figure 1D). The other pairwise comparisons were not significant. Also, for mixed biofilms, the number of CFUs of *C. glabrata* was significantly higher when compared to that of *C. albicans*, regardless of the glucose supplementation and culture medium.

3.2 | Biomass quantification

For the association of *C. albicans* ATCC 10231 with *C. glabrata* ATCC 90030 (Figure 2A), the total biomass of the dual-species biofilm developed in SDB medium was significantly higher than that observed for each single biofilm, regardless of the glucose supplementation. Similarly, the biomass of mixed biofilms of *C. albicans* ATCC 10231 with *C. glabrata* ATCC 90030 in RPMI 1640 (Figure 2A), *C. albicans* ATCC 10231 with *C. glabrata* D1 in both culture media (Figure 2B), and *C. albicans* 324LA/94 with *C. glabrata* D1 in RPMI (Figure 2D) was significantly higher when compared to that for single biofilms of *C. glabrata*, and not significantly different from that of *C. albicans* in single cultures. Conversely, for the associations of *C. albicans* 324LA/94 with *C. glabrata* ATCC 90030 (Figure 2C) in both media, and *C. albicans* 324LA/94 with *C. glabrata* D1 in SDB (Figure 2D), the resulting biomass of mixed biofilms was significantly lower in comparison with that of single biofilms of *C. albicans*, and significantly higher than that of single biofilms of *C. glabrata*.

The supplementation of the media with glucose significantly influenced biofilm biomass for the four microbial associations. For mixed biofilms of *C. albicans* ATCC 10231 with *C. glabrata* ATCC 90030 (Figure 2A), and *C. albicans* ATCC 10231 with *C. glabrata* D1 (Figure 2B), the biomass was significantly higher for both culture media supplemented with 1% and 5% glucose, compared with media without additional glucose. For *C. albicans* 324LA/94 and *C. glabrata* ATCC 90030 (Figure 2C), and *C. albicans* 324LA/94 and *C. glabrata* D1 (Figure 2D), in single and mixed cultures, the overall trend was that the biomass was significantly higher for biofilms formed in SDB supplemented with 1% and 5% glucose, when compared with no additional glucose. Finally, comparing both culture media as a function of glucose supplementation, significant differences were observed between the media supplemented with 1% and 5% glucose, for single- and dual-species biofilms of *C. albicans* ATCC 10231 and *C. glabrata* ATCC 90030 (Figure 2A), with a tendency of higher biomass for biofilms formed in SDB. For *C. albicans* ATCC 10231 and *C. glabrata* D1 (single and mixed biofilms, Figure 2B), a similar pattern was observed only for the media supplemented with 1% glucose.

3.3 | Evaluation of the metabolic activity

The results of XTT reduction are displayed in Figure 3. The metabolic activity of dual-species biofilms did not significantly differ from that found for single biofilms, except for mixed biofilm of *C. albicans* ATCC 10231 with *C. glabrata* ATCC 90030 (Figure 3A), which was significantly higher when compared to single biofilm of *C. glabrata* ATCC 90030 in SDB, and significantly lower when compared to the same biofilm in RPMI 1640, both supplemented with 1% glucose. The most evident pattern was that the metabolic activity of biofilms developed in SDB medium without glucose supplementation was markedly reduced when compared to the other conditions (media and glucose supplementation). Similarly, lower metabolic activity was observed for biofilms developed in RPMI 1640 medium without additional glucose when compared to the same medium supplemented with 1% or 5% glucose, despite a less marked trend was observed, and only applied for some pairwise comparisons. It was also observed significantly higher metabolic activity of biofilms formed in RPMI 1640 in comparison with SDB for most of the comparisons, since glucose supplementation also influenced the results.

3.4 | Biofilm structure

Scanning electron microscopy image of *C. albicans* ATCC 10231 biofilm grown in SDB supplemented with 1% (Figure 4A) and 5% (Figure 4G) glucose showed multilayers of yeasts and some hyphae covering the polystyrene surface, while single biofilm of *C. albicans* ATCC 10231 formed in RPMI 1640 supplemented with glucose at 1% (Figure 4D) had a more compact structure and a higher number of hyphae when compared with SDB (Figure 4A and G). Biofilms of *C. glabrata* ATCC 90030 and D1 formed in SDB supplemented with glucose at 1% (Figure 4B) and 5% (Figure 4H), and RPMI 1640 at 1% glucose (Figure 4E) were only constituted of multilayers of yeasts, partially covering the surface. On the other hand, mixed biofilms of *C. albicans* ATCC 10231 and *C. glabrata* ATCC 90030 formed in SDB supplemented with glucose at 1% (Figure 4C), and *C. albicans* ATCC 10231 and *C. glabrata* D1 formed in SDB at 5% glucose (Figure 4I) had a similar structure of that for single biofilms of *C. albicans* ATCC 10231 (Figure 4A and G), except that the presence of hyphae was more evident in mixed biofilms. Conversely, dual-species biofilm of *C. albicans* ATCC 10231 and *C. glabrata* ATCC 90030 (Figure 4F) revealed a less dense structure compared with the above-mentioned mixed biofilms (Figure 4C and I).

In turn, *C. albicans* 324LA/94 biofilm formed in SDB (Figure 4J) or RPMI 1640 (Figure 4M) without additional glucose was composed by a dense hyphae network, with few or no visible yeasts, respectively.

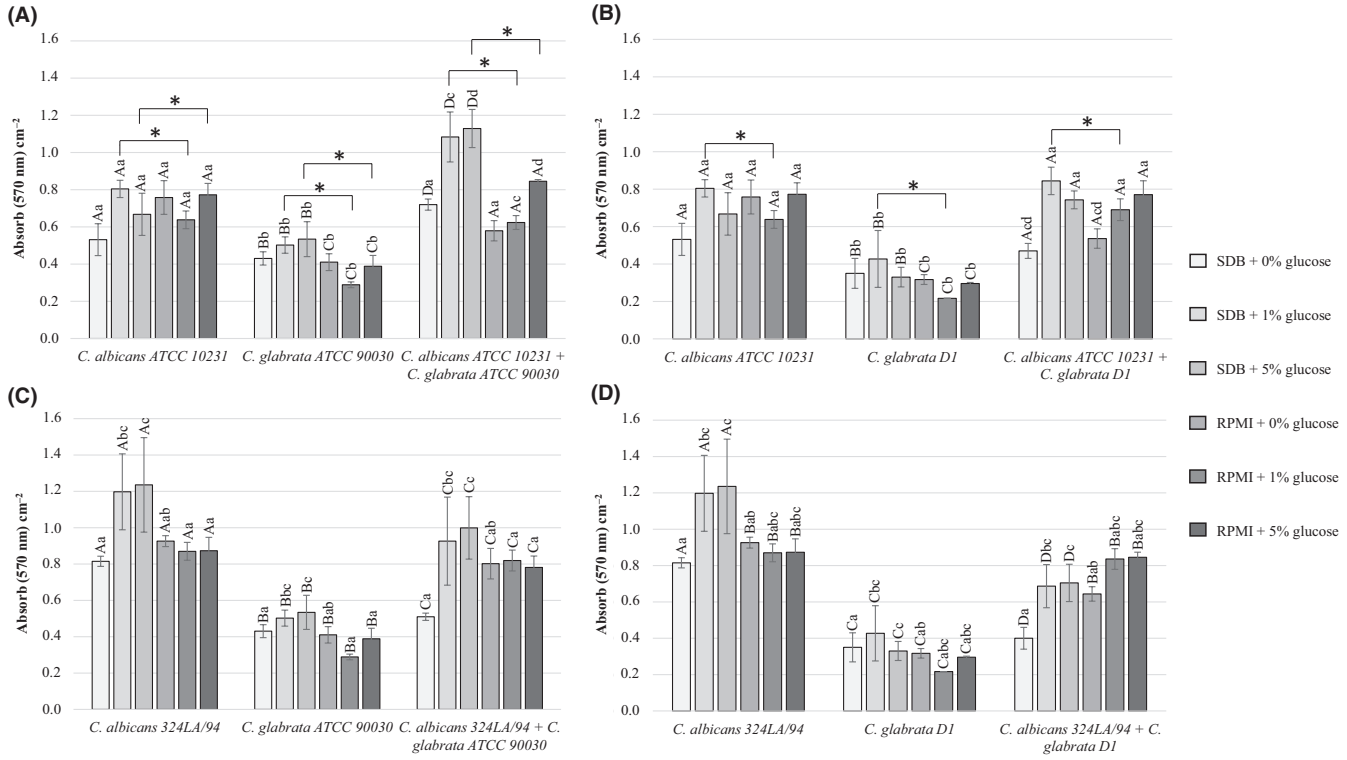

FIGURE 2 Mean absorbance values obtained from the total biomass quantification assay for the interactions between *Candida albicans* and *Candida glabrata* in biofilms. For the microbial associations shown in (A) and (B), letters indicate significant differences for the interactions between strain and medium (upper case), and strain and glucose supplementation (lower case), while asterisks represent significant differences for the interaction between medium and glucose supplementation. For the association presented in (C), letters indicate significant differences between strains (upper case) and for the interaction between medium and glucose supplementation (lower case). For the association shown in (D), letters indicate significant differences for the interaction between strain and medium (upper case), and between medium and glucose supplementation (lower case). Three-way ANOVA and Tukey's HSD post-hoc test, $P < .05$

For biofilms of *C. glabrata* D1 formed on the same media described above (Figure 4K and N, respectively), compact structures of multilayers of yeasts fully covering the surface were noted. For mixed biofilms of the above-mentioned species (Figure 4L and O), a lower number of hyphae and a higher number of yeasts were observed when compared with single biofilms of *C. albicans* 324LA/94 (Figure 4J and M). Also, a higher number of hyphae was observed for mixed biofilm of *C. albicans* 324LA/94 and *C. glabrata* D1 formed in RPMI 1640 without additional glucose (Figure 4O) compared with SDB medium (Figure 4L).

4 | DISCUSSION

Studies of interactions between different microorganisms are extremely important as they allow a better understanding about the pathogenicity of the species involved and may help in the development of alternative therapies to combat them.[23] Thus, the present study evaluated the interactions between *C. albicans* and *C. glabrata* in mixed biofilms formed in vitro, varying the type of strain (reference and oral clinical isolate), culture medium (SDB and RPMI 1640) and glucose supplementation (0%, 1% and 5%).

Regarding the number of CFUs, despite most of the microbial interactions was antagonistic to *C. albicans* and indifferent to *C. glabrata* in SDB, all microbial associations in RPMI 1640 were classified as indifferent for both species (Figure 1A-D). Barros et al[23] and Rossoni et al[24] observed reductions of 56% and 77% in the CFU values of *C. albicans* in mixed culture with *C. glabrata*, respectively, when compared to single biofilms. For these authors, *C. glabrata* may alter or inhibit the mechanism involved in the in vitro adherence of *C. albicans* in mixed biofilms, influencing the pathogenicity of this species and suggesting a competitive interaction for space and nutrients during biofilm formation. Interestingly, while dual-species biofilms of *C. albicans* ATCC 10231 with *C. glabrata* ATCC 90030 in SDB resulted in biomass significantly higher than that observed for each single biofilm (Figure 2A), an antagonistic effect on CFU enumeration was seen for the mixed biofilm when compared with single biofilm of *C. albicans* (Figure 1A). Taken together, these findings show that the increase in the biomass noted for the mixed biofilm might be related to the higher extracellular matrix in dual-species biofilm, and/or to the higher number of hyphae in comparison with single biofilms of *C. albicans* (as suggested in Figure 4A and C), considering that hyphae are elongated structures that absorb higher quantities of CV than yeasts.

The total biomass quantification revealed that the interactions between *Candida* species were affected by strain type, culture media and glucose supplementation (Figure 2), with different interactions among the variables depending on the strains involved. For the associations of

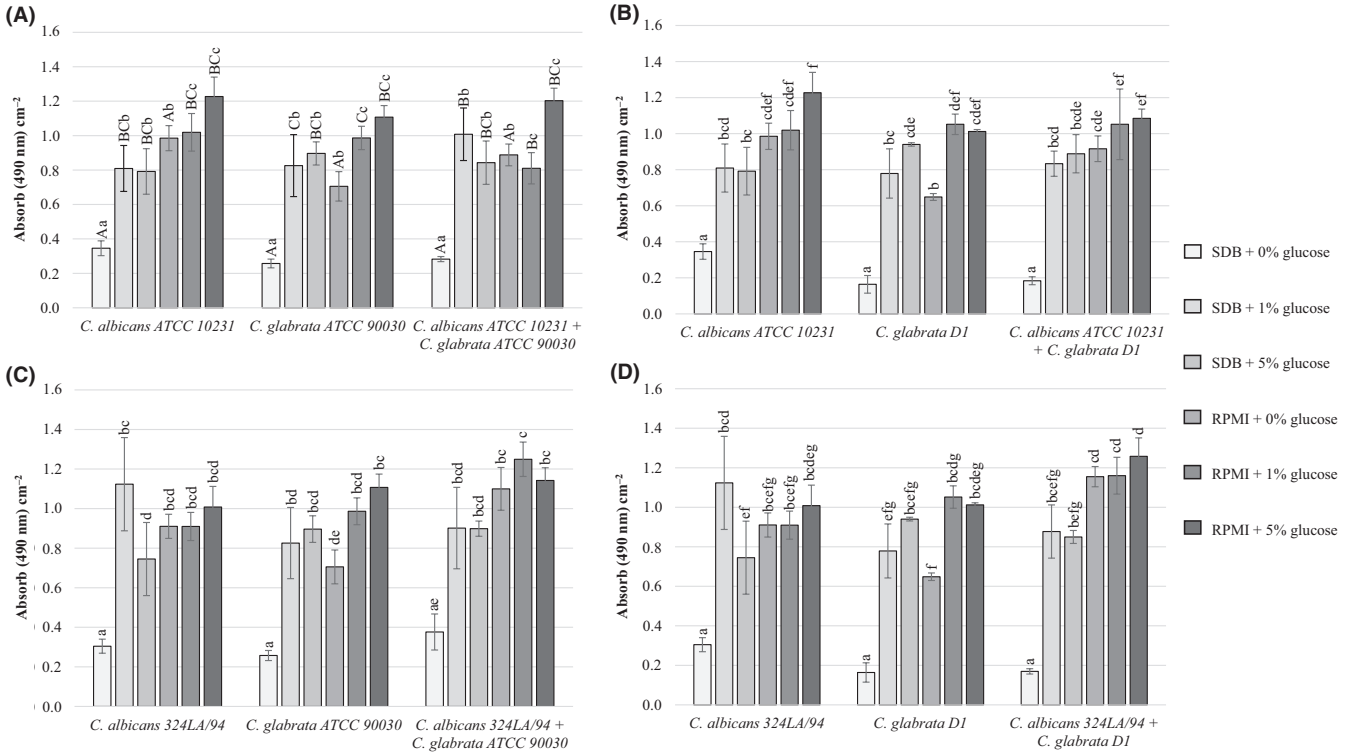

FIGURE 3 Mean absorbance values obtained from the XTT reduction assay for the interactions between *Candida albicans* and *Candida glabrata* in biofilms. For the microbial association displayed in (A), letters indicate significant differences for the interactions between strain and glucose supplementation (upper case) and between medium and glucose supplementation (lower case). For the associations shown in (B), (C) and (D), lower case letters denote significant differences for the interactions of all study variables (strain, medium and glucose supplementation). Three-way ANOVA and Tukey's HSD post-hoc test, $P < .05$

C. albicans 324LA/94 with *C. glabrata* ATCC 90030 (in SDB or RMPI), and *C. albicans* 324LA/94 with *C. glabrata* D1 (in SDB), the mixed biofilm biomass was higher in comparison to *C. glabrata* alone, but significantly lower than that of *C. albicans*. These results suggest a possible competition between these species, with a reduction in the development of *C. albicans* 324LA/94 in the presence of both strains of *C. glabrata*. The results of cultivable cells confirm these findings, since a lower number of *C. albicans* 324LA/94 cells in mixed culture with *C. glabrata* ATCC 90030 or D1 was observed when compared with the single biofilm, mainly in SDB (Figure 1C and D). For all other microbial associations, biomass of dual-species biofilms was not significantly different from single biofilms of *C. albicans*, but significantly higher in comparison with single biofilms of *C. glabrata*. As the number of cells in mixed biofilms was almost two-fold higher than that found in mono-species biofilms (Figure 1), it can be deduced that *C. albicans* produced greater amount of extracellular matrix when in single culture. These findings seem to explain the absence of synergism taking into account the assumed criterion to classify the microbial interactions.

Biofilms formed by the reference strains and clinical isolates also showed varied production of total biomass. In general, *C. albicans* resulted in biofilms with larger amount of total biomass than *C. glabrata*, regardless of the culture medium and glucose supplementation. These results are in line with SEM observations (Figure 4), which show structural differences between biofilms of these species. While *C. glabrata* biofilms are only composed of yeasts and produce lower amount of exopolymeric material,[25] biofilms of *C. albicans* present intermixed composition (yeasts of higher diameter, pseudohyphae and hyphae), what promotes a higher CV absorption when compared with *C. glabrata* which, in turn, results in higher absorbance values. Similarly, the amount of biofilm biomass of *C. albicans* 324LA/94 was higher than that originated by *C. albicans* ATCC 10231, under all experimental conditions, and SEM observations clearly show a higher number of hyphae for biofilms of *C. albicans* 324LA/94 when compared with the reference strain (Figure 4A, D, G, J and M). Furthermore, different strains present differences in their ability to form biofilms[26] due to the biological variability between strains of the same species or between different species. Nevertheless, in mixed biofilms, the total biomass may differ from that found for each single biofilm, depending on the type of relationship established between the microorganisms, as demonstrated in the present study. Rossoni et al[24] used only reference strains to evaluate the interactions among *C. albicans*, *C. krusei* and *C. glabrata*, and emphasised the importance of the use of clinical isolates, since previous studies have demonstrated intra-species variability for clinical strains of *Candida* in relation to the capacity of biofilm formation and pathogenicity in animal models.[27-29] Also, Pathak et al[30] found higher biofilm production (24 hours) by *C. glabrata* on acrylic surfaces when compared to *C. albicans*, with a higher biomass when these species were associated.[30] The differences between the results of the present study and those of Pathak et al[30] may be

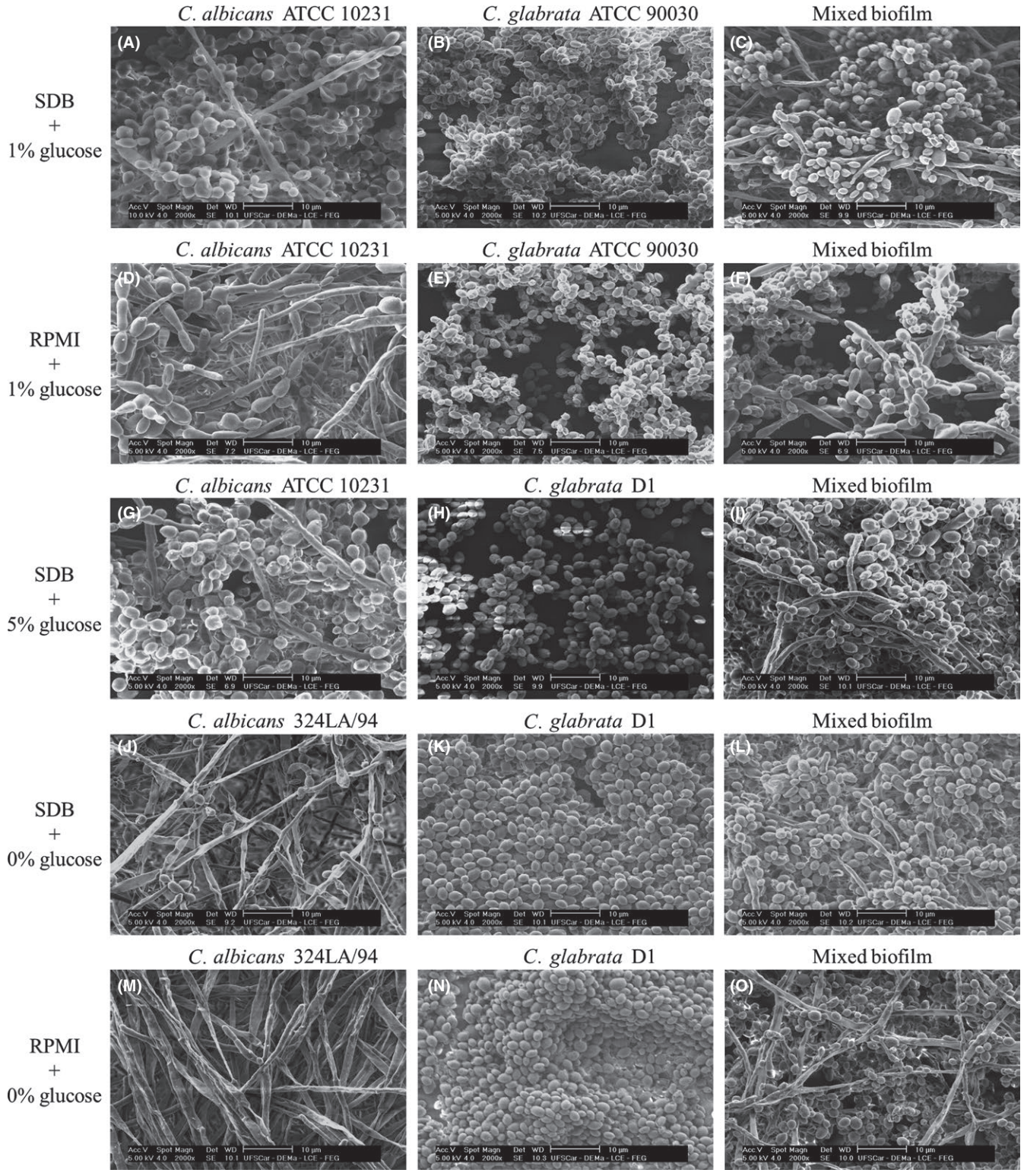

FIGURE 4 Scanning electron microscopy images of single- and dual-species biofilms of *Candida albicans* ATCC 10231, *Candida glabrata* ATCC 90030, *C. albicans* 324LA/94 and *C. glabrata* D1, grown in SDB or RPMI 1640 media, supplemented with glucose at 0%, 1% or 5%. Magnification: ×2000. Bars: 10 μm. ATCC, American Type Culture Collection; SDB, Sabouraud dextrose broth

associated with the surface and time for biofilm formation, as well as with the type of strain tested.

Although there were increases or decreases in the number of CFUs (Figure 1) and total biomass (Figure 2) for some microbial associations, the absorbance values obtained with the XTT reduction assay for mixed biofilms did not significantly differ from those found for single biofilms, except for the dual-species biofilm of *C. albicans* ATCC 10231 and *C. glabrata* ATCC 90030. For this biofilm, significantly higher and

lower metabolic activities were observed when compared with single biofilms of *C. glabrata* formed in SDB and RPMI 1640, respectively, both supplemented with 1% glucose (Figure 3A). Analysing these findings in conjunction with those of CFU, it is possible to infer that the metabolic activity of mixed biofilm cells was lower than that observed for the single ones, considering the higher number of cells present in dual-species biofilms (Figure 1).

Concerning the influence of culture media, the significant differences seen between SDB and RPMI 1640 for some microbial interactions indicated higher CFU and CV values for biofilms formed in SDB (Figures 1 and 2). Contrarily, significantly higher metabolic activity was observed for biofilms formed in RPMI 1640 (Figure 3). In fact, conflicting evidence on this subject is available on the literature. Tan et al[31] found higher metabolic activity (XTT assay) and biofilm formation (CV assay) for non-*albicans Candida* species in biofilms formed in Yeast Peptone Dextrose and Brain Heart Infusion media in comparison with RPMI 1640. On the other hand, Weerasekera et al[32] reported that RPMI 1640 promoted a significantly higher biofilm formation (measured by MTT and CV assays) than SDB for *C. albicans* and *Candida tropicalis*. A direct comparison of our results with the above-mentioned studies is not possible due to differences in the strains, culture conditions and quantification methods. In this sense, despite RPMI has been suggested as the medium of choice for future experiments, thus allowing cross comparison among different studies,[32] the lack of CFU data on the above-mentioned study, along with other important variables (eg, glucose supplementation and type of strain), indicate that this important question remains unanswered.

Interestingly, SEM images suggest a higher number of hyphae for biofilms of *C. albicans* ATCC 10231 formed at 1% glucose in RPMI 1640 (Figure 4D) in comparison with SDB (Figure 4A). A similar tendency was observed for single biofilms of *C. albicans* 324LA/94 in RPMI (Figure 4M) compared with SDB (Figure 4J), and for this species in co-culture with *C. glabrata* D1 (Figure 4O and L). Previous data support these observations, as RPMI 1640 was shown to have a higher potential to induce yeast-to-hyphae transformation.[33]

It is believed that the role of the dietary sugars is related to the modulation of the yeast growth rate and to cell wall components.[18] Thus, to examine the influence of the glucose supplementation on the formation of *Candida* biofilms, as well as on the interactions between different species, the concentrations of 0%, 1% and 5% were approximately 0, 0.5- and 3-fold of that used by Jin et al,[18] to mimic glucose-poor and -rich environments. This variable was also assessed to study the influence of variations of glucose concentrations in the oral cavity, which occur as a function of intake of foods and beverages with different carbohydrate concentrations.

In general, the results of the present study showed that when the addition of glucose influenced the CV and XTT results, higher biomass and metabolic activity was seen for both culture media. This emphasises that higher amount of energy promoted by glucose-rich environments increase the metabolic activity of the biofilm cells. It is noteworthy that the increase in the glucose concentration from 1% to 5% was not enough to promote a marked effect on the biofilm formation. In this sense, the lower metabolic activity of biofilms formed without additional glucose may be associated with a diminished extracellular matrix production, thus reflecting in reduced biofilm biomass, as observed for biofilms formed in SDB without additional glucose (Figure 2) seen for most of the microbial interactions. Such pattern, however, was not observed for CFU enumeration data, given that the number of cultivable cells for biofilms formed without additional glucose was not significantly different from those supplemented with 1% and 5% glucose (Figure 1). These results are relevant from a clinical standpoint, as they highlight that even in environments with glucose deprivation *Candida* species are able to form biofilms.

Within the limitations of this in vitro study, it was possible to conclude that the type of strain, culture medium and glucose supplementation influenced the interactions between *C. albicans* and *C. glabrata*. Future studies involving other *Candida* species and different models of biofilm formation should be stimulated, aiming to a better understanding of the interactions and pathogenicity of *Candida* species in polymicrobial biofilms.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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