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Environmental pH modulates biofilm formation and matrix composition in *Candida albicans* and *Candida glabrata*

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ABSTRACT

Candida species are fungal opportunistic pathogens capable of colonizing and infecting various human anatomical sites, where they have to adapt to distinct niche-specific pH conditions. The aim of this study was to analyse the features of *Candida albicans* and *Candida glabrata* biofilms developed under neutral and vaginal acidic (pH 4) conditions. *C. albicans* produced thicker and more filamentous biofilms under neutral than under acidic conditions. On the other hand, the formation of biofilms by *C. glabrata* was potentiated by the acidic conditions suggesting the high adaptability of this species to the vaginal environment. In general, both species developed biofilms containing higher amounts of matrix components (protein and carbohydrate) under neutral than acidic conditions, although the opposite result was found for one *C. glabrata* strain. Overall, this study contributes to a better understanding of the modulation of *C. albicans* and *C. glabrata* virulence by specific pH conditions.

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Introduction

Candida species which are the most common yeasts found in the normal human microbiome are also capable of causing morbidity and mortality (Alberto Cortés and Fernanda Corrales 2019). Candidiasis is associated with a wide variety of clinical manifestations, ranging from mucosal infections with mild severity to life-threatening bloodstream infections (Pappas et al. 2016). *Candida albicans* is the leading cause of candidiasis but a trend to non-*Candida albicans Candida* species (NCAC), especially *Candida glabrata*, has been suggested by some epidemiologic surveys (Gonçalves et al. 2016; Alberto Cortés and Fernanda Corrales 2019). *C. glabrata* has high clinical relevance owing to its low susceptibility to some classes of antifungals and its ability to develop resistance following exposure to antifungal agents (Bennett et al. 2004; Nagashima et al. 2016).

The pathogenicity of *Candida* species is mediated by several virulence factors, including the ability to form biofilms on mucosal surfaces and indwelling medical devices (Harriott et al. 2010; Bouza et al. 2014). *C. albicans* forms thick, spatially organised complex biofilms comprised of a multilayered and intertwined network of blastospores, pseudohyphae and hyphae, partially embedded in an extracellular matrix (Seneviratne et al. 2009). Importantly, although readily formed in planktonic cultures, in the presence of specific nutritional cues, hyphae are a characteristic feature of *C. albicans* biofilms and required for their proper formation (Nobile et al. 2012; Gulati and Nobile 2016). The hyphae contribute to the architectural stability of biofilms by forming a scaffold that provides a robust support for the extracellular matrix as well as the blastopores, pseudohyphae and other hyphae (Seneviratne et al. 2009; Gulati and Nobile 2016). Nevertheless, studies on *C. glabrata*, which does not naturally form true hyphae, suggest that hyphal morphology is not a prerequisite for biofilm formation. Indeed, *C. glabrata* develops thin and patchy, rather compact biofilms, exclusively of blastospores embedded within an extracellular matrix (Seneviratne et al. 2009). The association of microorganisms into biofilms contributes to their survival under hostile environmental conditions (Donlan and Costerton 2002).

One of the main challenges faced by *Candida* species during commensal and pathogenic lifestyles is the wide range of environmental pH in host niches, from acidic (vagina and stomach) to neutral and slightly alkaline (bloodstream and gut) (Davis 2003; Vylkova

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et al. 2011). The environmental pH affects *Candida* cells, especially at their plasma membranes, including having effects on protein activity, proton gradients and nutrient availability (Davis 2003). Importantly, changes in the environmental pH can occur within some niches. For example, the vaginal tract has a normal pH of ~ 4 but this can increase to 5.5–7.5 during menstruation, pre-puberty or post-menopause (García-Closas et al. 1999) or in the presence of exogenous substances (Bouvet et al. 1997; Polatti et al. 2006). The vaginal tract is an interesting niche, because its acidity is a recognized barrier against most pathogens, but *Candida* species cause infection at its normal acidic pH (Linhares et al. 2011; Hickey et al. 2012). Previous studies have reported that an acidic pH has a significant impact on biofilm formation by *Candida* species (Kucharikova et al. 2011; Ferreira et al. 2016). However, the effect of specific vaginal acidity, which is mainly promoted by the production of lactic acid by the vaginal mucosa (L-lactate) and bacteria (L-lactate and D-lactate) (Boskey et al. 2001; Linhares et al. 2011), has not yet been investigated. Importantly, unlike other acids, lactic acid does not exert an inhibitory effect on *Candida* growth (Lourenço et al. 2019), contrasting with its effect on bacterial vaginal pathogens (O'Hanlon et al. 2011). As such, it is crucial to clarify the influence of vaginal acidic pH on *Candida* virulence factors, especially for the two most common species in the vaginal environment, *C. albicans* and *C. glabrata*. This study analysed relevant features of biofilms of *C. albicans* and *C. glabrata* developed under neutral and acidic conditions, the latter adjusted to pH 4 with lactic acid (L-lactate), as an approximation of the vaginal acidic environment. This knowledge will contribute to a better understanding of the modulation of *C. albicans* and *C. glabrata* biofilms by the vaginal acidity, providing relevant information for future studies of *Candida* vaginal virulence.

Materials and methods

Strains and initial culture conditions

In this study one reference strain and two vaginal isolates of *C. albicans* (SC5314, 557843 and 569322) and *C. glabrata* (ATCC 2001, 534784, 585626) were used. The isolates were supplied from the Biofilm Group collection (Centre of Biological Engineering, University of Minho, Braga, Portugal) and were isolated from patients at the Hospital of S. Marcos, Braga, Portugal. The identity of the isolates was confirmed using CHROMagar *Candida* (CHROMagar, Paris, France) and PCR-based identification with specific primers (ITS1 and ITS4) (Williams et al. 1995).

Candida strains were grown on Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) at 37 °C for 48 h. For each experiment, colonies collected from the SDA plates were used to inoculate 40 ml of Sabouraud dextrose broth (SDB; Merck, Darmstadt, Germany), which was incubated at 37 °C for 18 h under agitation (120 rev min^{-1}). The cell suspension was centrifuged at 5,000 g for 10 min at 4 °C and the cells were washed twice with 10 ml of sterile ultrapure water. The supernatant was discarded and the pellet was resuspended in Roswell Park Memorial Institute (RPMI-1640; Sigma-Aldrich, St Louis, Missouri) medium buffered with 3-(N-Morpholino) propanesulfonic acid (MOPS; Sigma-Aldrich, St Louis, Missouri). RPMI-1640 was used at pH 7 or pH 4 (adjusted with L-lactate; Merck, Darmstadt, Germany). For the planktonic and biofilm experiments the cell density was adjusted to 1×10^5 cells ml^{-1} using a Neubauer haemocytometer (Marienfeld, Lauda-Königshofen, Germany). The experiments described in the next sections were performed in triplicate (same pre-inoculum) and in three independent assays (pre-inocula independently prepared).

Analysis of planktonic growth and filamentation

For planktonic growth, cell suspensions of *C. albicans* and *C. glabrata* prepared in RPMI-1640 at pH 4 and 7 were placed in 25 ml Erlenmeyer flasks and incubated for 24 h at 37 °C under agitation in an orbital shaker (120 rev min^{-1}). Aliquots (200 µl) were collected from the cell suspensions over 24 h and their optical density was measured, at 600 nm, using a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Winooski, Vermont). The pH was monitored throughout the experiment to ensure that the desired value was maintained.

Additionally, the filamentation of *C. albicans* strains was evaluated after cell growth for 24 h. For this, an aliquot of cell suspension (200 µl) was diluted in sterile water (1:100) and the filamentous forms were counted using an optical microscope and the results presented as a percentage of filamentous forms.

Biofilm formation

In order to develop *C. albicans* and *C. glabrata* biofilms, the cell suspensions prepared in RPMI-1640 at pH 4 and 7 were placed in 96-well polystyrene

microtiter plates (Orange Scientific, Braine-lAlleud, Belgium) (200 µl per well), which were incubated for 24 h at 37 °C under agitation (120 rev min-[1]) (Silva et al. 2009). After incubation, the medium was removed and the biofilms were washed with 200 µl of Phosphate Buffered Saline (PBS; 0.01 M) to remove non-adherent cells. The pH was monitored throughout the experiments to ensure that the desired value was maintained. The biofilms were analysed as described in the next sections.

Quantification of biofilm biomass

The total biomass of biofilms was quantified using the Crystal violet (CV) staining methodology (Silva et al. 2009). For that, the biofilms developed were fixed with methanol (200 µl per well) for 15 min and then stained with CV (1% v v^{-1}; 200 µl per well) for 5 min. The excess dye was removed by washing the biofilms twice with sterile water. Finally, 200 µl of acetic acid (33%, v v^{-1}) were added to each well and the solutions were homogenized to release and dissolve the dye. The absorbance of the solutions was measured on a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Winooski, Vermont) at 570 nm and the results presented as absorbance per unit area (Absorbance CV cm^{-2}).

Determination of biofilm cell cultivability

The number of cultivable cells in the biofilms was estimated using the colony forming units (CFU) counting methodology (Silva et al. 2010). Briefly, the biofilms developed were scraped from the microtiter plate wells in the presence of 200 µl of PBS and the suspensions were vigorously vortexed for 2 min to disaggregate the cells (Silva et al. 2010). Serial 10-fold dilutions were prepared of the biofilm suspensions in PBS, which were plated onto SDA plates (10 µl) and incubated for 24 h at 37 °C. After incubation, the number of CFU on the SDA plates was counted and the results presented per unit area (Log CFU cm^{-2}).

Biofilm thickness analysis

A confocal laser scanning microscope (CLSM) was used to analyse the thickness of biofilms developed for 24 h, as previously described, but using 24-well polystyrene microtiter plates (Orange Scientific, Braine-lAlleud, Belgium) (1 ml per well). The biofilms were stained with 1% (v v^{-1}) Calcofluor white (Sigma-Aldrich, St Louis, Missouri) for 10 min at room temperature in the dark and then observed with a CLSM (Olympus BX61, Model FluoView 1000, Portugal). Excitation line 405 and emission filters BA 430–470 (blue channel) were used and images were acquired with the program FV10-ASW 4.2 (Olympus). The biofilm thickness was analysed in three areas of each image and the median thickness value was calculated for each replicate.

Analysis of filamentous forms in biofilm cells

The percentage of filamentous forms in the *C. albicans* biofilm cells, was determined from the biofilm suspensions, as previously described for planktonic cells. The filamentous forms of biofilm cells were also visualized with a CLSM, as described in the previous section.

Biofilm matrix extraction and analysis

In order to extract the biofilm matrix, *C. albicans* and *C. glabrata* biofilms were developed as described above, using 24-well polystyrene microtiter plates, and then scraped from the wells, resuspended in ultrapure water and sonicated (Ultrasonic Processor, Cole-Parmer, Vernon Hills, Illinois) for 30 s at 30 W (Silva et al. 2009). The suspensions were vortexed for 2 min and centrifuged at 5,000 g for 5 min. The pellets were dried at 37 °C until a constant weight was obtained. The matrix-containing supernatants were filtered through a 0.2 µm nitrocellulose filter and then the protein, total carbohydrate and (1,3)-β-D-glucan contents were estimated as described in the following sections.

Protein quantification

The protein content was measured using the BCA Kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, Missouri) and bovine serum albumin (BSA) as standard (Silva et al. 2009). Briefly, 0.2 ml of BCA solution were added to 25 µl of matrix sample and incubated for 30 min at 37 °C. Then, the absorbance was determined in a microplate reader at 562 nm. The protein concentration was extrapolated from a calibration curve ($abs = 0.009 \times [protein] + 0.1685$) performed with standard concentrations of BSA. The results were normalized with the dry weight of biofilm cells, previously determined, and presented as mg of protein per g of biofilm (mg $g_{biofilm}^{-1}$).

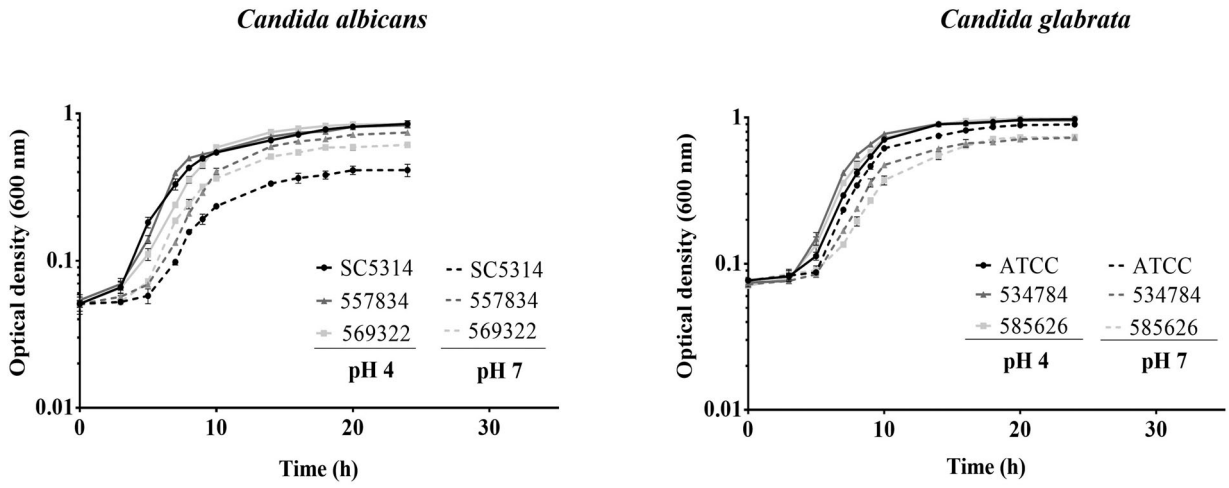

Figure 1. **Planktonic growth of *C. albicans* and *C. glabrata* at pH 4 and pH 7.** Growth curves (optical density) of *C. albicans* SC5314 (reference), 557834 and 569322 (vaginal isolates) and *C. glabrata* ATCC 2001 (reference), 537834 and 585626 (vaginal isolates), performed over 24 h at pH 4 and 7. Error bars represent SDs.

Carbohydrate quantification

The total carbohydrate content was estimated using the phenol-sulfuric method (DuBois et al. 1956) and glucose as standard. Briefly, 0.5 ml of phenol (50 g l^{-1}) and 2.5 ml of sulfuric acid (95–97%) were added to 0.5 ml of matrix sample. The solution was vortexed for 30 s and incubated for 15 min at room temperature. The absorbance was determined in a microtiter plate reader at 490 nm. The concentration of carbohydrate was extrapolated from a calibration curve (Abs = 0.2955 × [carbohydrate] + 0.114) performed with standard glucose concentrations. The results were normalized with the dry weight of biofilm cells and presented as mg of carbohydrate per g of biofilm (mg $g_{biofilm}^{-1}$).

(1,3)-β-D-glucan quantification

The concentration of (1,3)-β-D-glucan was determined using the Glucatell (1,3)-β-D-Glucan Detection Reagent kit (Associates of Cape Cod Inc., East Falmouth, Massachusetts). Briefly, 50 µl of Glucatell reagent were added to 50 µl of matrix sample and the solution was incubated at 37 °C for 30 min. The reaction was stopped by adding 50 µl of sodium nitrite and sequentially 50 µl of ammonium sulfamate and 50 µl of N-(1-Napthyl) ethylenediamine dihydrochloride. The optical density was read at 540 nm and the concentration of (1,3)-β-D-glucan was extrapolated from a calibration curve performed with standard glucan concentrations (Abs = 0.0106 × [glucans] + 0.0392). The results were normalized with the dry weight of biofilm cells and presented as ng of (1,3)-β-D-glucan per g of biofilm (ng $g_{biofilm}^{-1}$).

Statistical analysis

The results of biofilm biomass, cells cultivability, filamentation and matrix composition were analysed statistically using GraphPad Prism 6 software. The results obtained at pH 4 were compared with those obtained at pH 7 using the *t* test analysis. All tests were performed with a confidence level of 95%.

Results

Planktonic growth under acidic and neutral conditions

In order to analyse the influence of pH on the planktonic growth of *C. albicans* and *C. glabrata strains,* the optical density of free-floating cells growing in RPMI-1640 buffered to pH 4 and 7 was monitored for 24 h. The expected growth stages were obtained for all the *C. albicans* and *C. glabrata* strains under the conditions tested (Figure 1). The growth of *C. glabrata* strains was similar under both conditions, with the *C. albicans* strains having a slightly faster growth rate under acidic than neutral conditions. Additionally, the growth of the reference strain and the vaginal isolates was similar in both species under both conditions (Figure 1).

Biofilm formation under acidic and neutral conditions

In order to study the influence of pH on the formation of biofilms, biofilms were developed for 24 h in RPMI-1640 at pH 4 and 7, and analysed in terms of

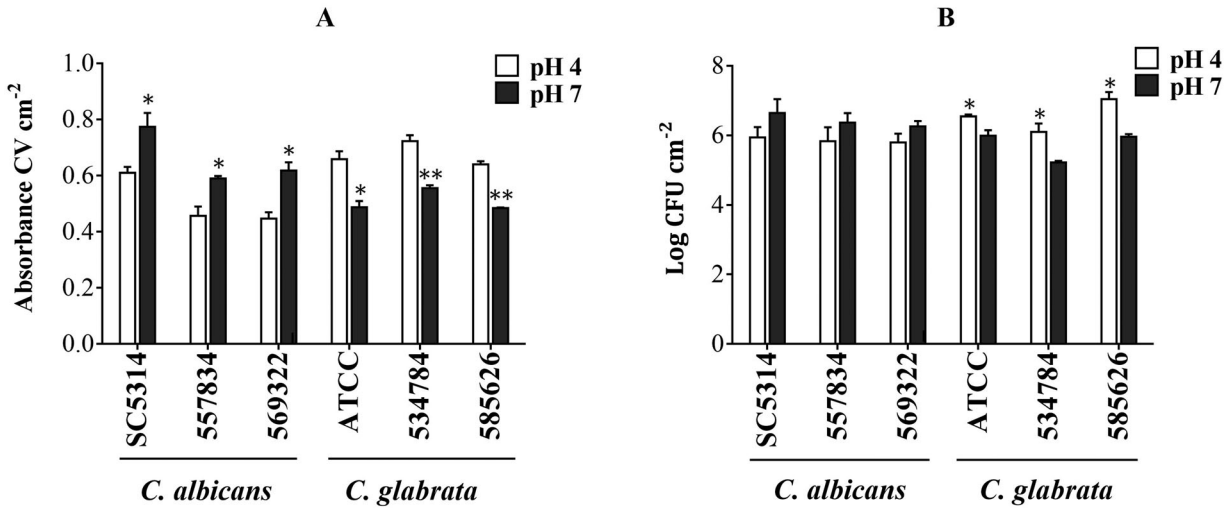

Figure 2. Biofilm formation by *C. albicans* and *C. glabrata* at pH 4 and pH 7. (A) Total biomass quantification (Absorbance CV cm^{-2}) and **(B)** cultivable cell determination (Log CFU cm^{-2}) of *C. albicans* SC5314 (reference), 557834 and 569322 (vaginal isolates) and *C. glabrata* ATCC 2001 (reference), 537834 and 585626 (vaginal isolates) biofilms, developed over 24 h at pH 4 and pH 7. Error bars represent SDs; asterisks represent statistical differences between the results obtained at pH 4 and pH 7 (** p-value $\leq$ 0.01; * p-value $\leq$ 0.05).

total biomass (Figure 2A) and the number of cultivable cells (Figure 2B). All strains of *C. albicans* and *C. glabrata* formed biofilms under both conditions, with pH having a different effect on each species (Figure 2). A statistically lower amount of biomass (p-value $\leq$ 0.05) was found in biofilms of *C. albicans* at pH 4 than at pH 7, with an average difference between the conditions of 20% (Figure 2A). Consistently, *C. albicans* biofilms developed at pH 4 contained fewer cultivable cells than those formed at pH 7, although the difference was not statistically significant (p-value $>$ 0.05) (Figure 2B).

In contrast, a greater amount of biomass (p-value $\leq$ 0.01) and a higher number of cultivable cells (p-value $\leq$ 0.05) were found in biofilms of *C. glabrata* at pH 4 than at pH 7. The average difference between the conditions was 24% (Figure 2A) and 1 order of magnitude (Log CFU (cm^{-2}) (Figure 2B) for total biomass and cell cultivability, respectively.

C. albicans and *C. glabrata* biofilms were further analysed with a CLSM in order to determine their thickness. *C. albicans* biofilms formed at pH 4 were thinner than biofilms formed at pH 7 (Table 1 and Supplementary Figure S1), consistent with the previous analyses (Figure 2). In contrast, and also in accordance with the previous results (Figure 2), *C. glabrata* biofilms formed at pH 4 were thicker (p-value $\leq$ 0.05) than biofilms formed at pH 7 (Table 1 and Supplementary Figure S2).

Table 1. Effect of pH on the biofilm thickness of *C. albicans* and *C. glabrata*.

		Biofilm thickness (μm)	
Species	Strain	pH 4	pH 7
C. albicans	SC5314	15.5 ± 1.2	28.3 ± 1.5**
	557834	13.4 ± 0.9	16.1 ± 1.7
	569322	13.0 ± 2.9	20.0 ± 3.1
C. glabrata	ATCC 2001	18.2 ± 1.8	12.3 ± 2.1*
	534784	25.5 ± 2.2	15.6 ± 2.4**
	585626	21.2 ± 2.5	13.8 ± 3.3*

Thickness (μm) of *C. albicans* and *C. glabrata* biofilms developed for 24 h at pH 4 and pH 7. Asterisks represent a statistical difference between the results obtained at pH 4 and pH 7 (** p-value $\leq$ 0.01; * p-value $\leq$ 0.05). The images of biofilms obtained by CLSM are available in Supplementary Figures S1 and S2.

Effect of pH on C. albicans filamentation

C. albicans strains grown in the planktonic and biofilm lifestyles were analysed by optical microscopy in order to evaluate the effect of pH on the development of filamentous forms (Figure 3). A higher percentage of filamentous forms was found at pH 7 than at pH 4, independent of the growth style (Figure 3A). The percentage of filamentous forms was statistically higher (p-value $\leq$ 0.05) under neutral than under acidic conditions for *C. albicans* SC5314 and 585626. These two strains formed $\sim$ 50% and 10% more filaments at pH 7 than at pH 4, respectively (Figure 3A). *C. albicans* 557834 showed a poor ability to produce filaments but a higher percentage of filaments was also found at pH 7 than at pH 4.

The length of the filamentous forms formed by biofilm cells was evaluated by selecting CLSM images from a zone of the biofilm in which the cells with filaments were visible at high resolution (Figure 3B). More filaments with a longer length were found at pH 7 than at pH 4 for *C. albicans* SC5314 and 569322. In *C. albicans* 557834 filaments were not

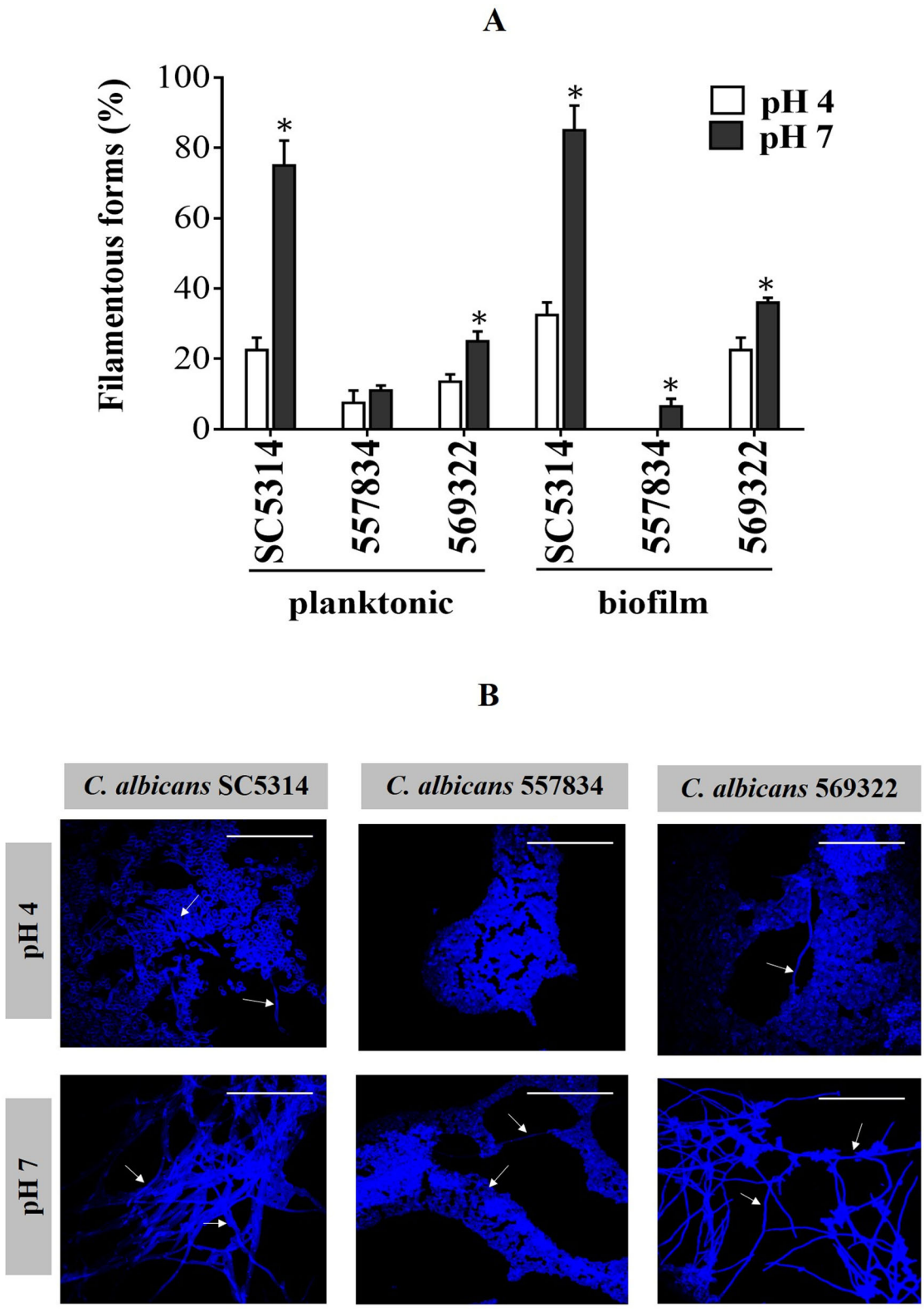

Figure 3. Effect of pH on filamentous form development in *C. albicans*. **(A)** Percentage of filamentous forms of *C. albicans* SC5314, 557834 and 569322 cells grown in planktonic and biofilm lifestyles for 24 h at pH 4 and pH 7. Error bars represent SDs; asterisks represent statistical differences between the results obtained at pH 4 and pH 7 (* p-value $\leq$ 0.05). **(B)** CLS images of *C. albicans* biofilm cells grown at pH 4 and 7. Arrows point to filamentous forms. Original magnification = 60 x; bar = 100 µm.

Table 2. Influence of pH on the biofilm matrix composition of *C. albicans* and *C. glabrata*.

Species	Strain	pH	Protein (mg $g_{biofilm}^{-1}$)	Carbohydrate (mg $g_{biofilm}^{-1}$)	(1,3)-β-D-glucan (ng $g_{biofilm}^{-1}$)
C. albicans	SC5314	**4**	42.92 ± 5.11	148.48 ± 11.37	47.47 ± 1.43
		7	64.31 ± 11.39	578.20 ± 10.26***	73.65 ± 6.57*
	557834	**4**	56.39 ± 7.27	398.48 ± 13.16	50.40 ± 2.94
		7	65.00 ± 6.09	889.20 ± 12.79***	75.47 ± 3.40*
	569322	**4**	50.69 ± 7.07	123.94 ± 5.38	54.00 ± 2.84
		7	77.36 ± 6.68	839.93 ± 7.25****	78.34 ± 5.70*
C. glabrata	ATCC 2001	**4**	37.68 ± 8.64	104.23 ± 13.4	45.91 ± 1.16
		7	73.89 ± 4.52 *	511.42 ± 6.58***	83.82 ± 2.94**
	534784	**4**	57.92 ± 1.18	99.83 ± 11.96	62.79 ± 6.62
		7	59.74 ± 6.1	772.42 ± 6.78***	89.75 ± 2.28*
	585626	**4**	55.28 ± 6.87	296.39 ± 5.18	55.73 ± 3.35
		7	29.31 ± 7.46	167.94 ± 16.15**	41.02 ± 0.42*

Amount of protein, carbohydrate and (1,3)-β- D-glucan in the matrix of *C. albicans* and *C. glabrata* biofilms developed at pH 4 and pH 7. Asterisks represent a statistical difference between the results obtained at pH 4 and pH 7 (**** p-value $\leq$ 0.0001; *** p-value $\leq$ 0.001; ** p-value $\leq$ 0.01; * p-value $\leq$ 0.05).

observed in the biofilms formed at pH 4 and those seen at pH 7 were shorter than those seen with the other strains (Figure 3B and Supplementary Figure S1).

Influence of pH on biofilm matrix composition

The matrix of *C. albicans* and *C. glabrata* biofilms was analysed to evaluate the influence of pH on the amounts of the major components, namely protein and carbohydrate (total content and (1,3)-β-D-glucan) present. The results showed that the pH significantly affected the composition of the biofilm matrix in both species (Table 2).

In all *C. albicans* strains, a higher amount of protein and carbohydrate was found in the matrix of biofilms formed under neutral compared with acidic conditions (Table 2). In *C. albicans* 569322, the amount of total carbohydrate measured at pH 7 was ~ six times higher than that measured at pH 4, and in the reference strain, it was almost four times higher than under neutral conditions (Table 2). Consistently, a statistically higher (p-value $\leq$ 0.05) amount of (1,3)-β-D-glucan was found in the matrix of C. *albicans* biofilms formed under neutral than under acidic conditions (Table 2). The content of this component found at pH 7 was ~ 1.5 times than that found at pH 4, in all strains. Additionally, a higher protein content was also found in the matrix of *C. albicans* biofilms formed under neutral conditions, but the difference in relation to the acidic environment was not statistically significant (p-value $>$ 0.05).

In contrast with *C. albicans*, the influence of pH on the composition of the *C. glabrata* biofilm matrix was not similar for all strains. The amount of carbohydrate and protein found in the matrix of *C. glabrata* 585626 biofilms formed at pH 7 was almost half of that found at pH 4 (Table 2). In comparison, the amount of carbohydrate found in the matrix *C. glabrata* 534784 biofilms at pH 7 was ~ seven times higher than that found at pH 4, and in the reference strain the amount was ~ five times higher under neutral conditions (Table 2). Consistently, a statistically higher (p-value $\leq$ 0.05) amount of (1,3)-β-D-glucan and a higher protein content were found in the matrix of the biofilms formed at neutral than in acidic conditions (Table 2).

Discussion

The ability of *Candida* species to adapt to the environmental pH of host niches is important for their success as commensals and as pathogens (Davis 2003). Importantly, the vaginal acidic pH is promoted by lactic acid, which has a particular non-inhibitory effect on *Candida* growth (Lourenço et al. 2019). As such, this study aimed to investigate the features of *C. albicans* and *C. glabrata* biofilms (reference strains and vaginal isolates) developed under a condition mimicking vaginal acidity (pH 4, with lactic acid), and compare them with those formed in a neutral environment. This study revealed a relevant and species-specific modulation of *Candida* biofilms by the vaginal acidity.

C. albicans developed biofilms with less biomass under acidic than under neutral conditions (Figure 2A), although the biofilm cell cultivability was not significantly affected (Figure 2B). Fewer and shorter filamentous forms were found in biofilms formed at pH 4 than at pH 7 (Figure 3 and Supplementary Figure S1), in line with the lower biomass found under acidic conditions (Figure 2A). Importantly, the reduced filamentation of *C. albicans* in an acidic pH has been suggested to facilitate the dispersion of biofilm cells displaying potentiated virulence traits, thus

contributing to the establishment of new foci of infection (Uppuluri et al. 2010). This is especially relevant for women using intrauterine devices, to which *Candida* species can easily adhere and form biofilms, becoming a source of highly virulent pathogens (Chassot et al. 2008).

Besides reduced filamentation, *C. albicans* biofilms developed under acidic conditions produced lower amounts of matrix components, especially carbohydrates, than those formed at neutral pH (Table 2). To the authors' knowledge this is the first study regarding the influence of pH on the *Candida* biofilm matrix. A predominant role has been demonstrated for matrix carbohydrates in the resistance of *C. albicans* biofilms to antifungal agents (Nett et al. 2010; Dominguez et al. 2018). In accordance, lower resistance to caspofungin was reported for *C. albicans* biofilms formed under acidic than under neutral conditions (Kucharikova et al. 2011). Caspofungin exerts its activity by inhibiting (1,3)-β-D-glucan synthases (Andriole 2000) and consistently, a lower content of (1,3)-β-D-glucan was found in the matrix of *C. albicans* biofilms formed under an acidic than under a neutral pH (Table 2).

In contrast to *C. albicans*, *C. glabrata* formed thicker biofilms (Table 1), with higher cell cultivability (Figure 2B), under acidic than under neutral conditions. The promotion of *C. glabrata* biofilm formation by the acidic conditions used in this study suggests that this species may exhibit high adaptability to the acidic vaginal environment. Strains of *C. glabrata* have commonly been isolated from women with recurrent vulvovaginal candidiasis (Grigoriou et al. 2006; Amouri et al. 2011). Recurrent episodes are suggested to result from the high ability of *Candida* species to adapt and form biofilms in the vaginal environment, making it difficult to eradicate this pathogen from the vaginal lumen (Sobel 2007; Harriott et al. 2010). Interestingly, proteomic analyses performed by Schmidt *et. al* (2008) suggested that *C. glabrata* perceives a low pH as less stressful than a higher pH, what is consistent with the idea that this species is an opportunistic pathogen of humans that has evolved primarily in relatively acidic environmental niches such as root fruit (Schmidt et al. 2008).

Unlike *C. albicans*, the influence of pH on *C. glabrata* biofilm matrix, was not consistent among strains. In *C. glabrata* 585626 the production of a biofilm matrix was higher under acidic than under neutral conditions, but in the reference strain and *C. glabrata* 534784 the opposite result was found (Table 2). These results suggest that a higher production of biofilm matrix under neutral conditions may be a mechanism of protection in response to a more stressful environment for *C. glabrata* strains (Schmidt et al. 2008), but this mechanism must be dependent on the strain.

Importantly, the results bring new insights into the modulation of *C. albicans* and *C. glabrata* biofilms by the specific vaginal acidity (with lactic acid), This study contrasts with that of Kucharikova et al. (2011), who found lower biofilm formation at acidic (pH 5.6 and hydrochloric acid) compared with neutral conditions, in both species. Unlike other acids, lactic acid has been shown to exhibit no inhibitory effect on *C. albicans* and *C. glabrata* growth, even at high concentrations (Lourenço et al. 2019), thus in line with the results obtained in the current study (Figures 1 and 2). The tolerance of *Candida* species to lactic acid is attributed to their rapid ability to utilize it for their metabolism, which is suggested to favour *Candida* species by improving their metabolic versatility in niches often deprived of glucose such as the vaginal tract (Childers et al. 2016; Lourenço et al. 2019). This study suggests that *Candida* species exhibit a specific adaptation to the vaginal acidity, and thus, acidic experiments with acids other than lactic may not be suitable for extrapolation to the vaginal environment. Furthermore, this study shows relevant differences between the response of *C. albicans* and *C. glabrata* biofilms to the environmental pH that could have significance with respect to their response to drugs and host defences, hampering the management of *Candida* infections. Importantly, the results shown for *C. albicans* suggest that a higher concentration of drugs and/or longer treatment time may be necessary to effectively treat biofilm-related infections of this species in a neutral environment, such as the bloodstream, than in the acidic vaginal environment. Moreover, the vaginal environment may become temporarily or permanently neutral under certain conditions, owing to an impaired production of lactic acid (García-Closas et al. 1999; Polatti et al. 2006). Thus, vaginal pH seems to be a relevant factor to consider when applying a treatment for vulvovaginal candidiasis caused by *C. albicans*. Additionally, the findings suggest that inherent physiological differences between *C glabrata* strains may lead to different biofilm responses to drugs under similar environmental pH conditions. Future studies, with higher numbers of isolates, will be crucial to investigate an explicit strain-dependency in *C. glabrata* and to definitely confirm the end-consequences indicated by this study.

The ability of *Candida* species to adapt to different environmental pH conditions is crucial for their

survival at the mucosa and on medical devices. This study revealed, for the first time, the modulation of *C. albicans* and *C. glabrata* biofilms by vaginal acidity, leading to a better understanding of the adaptation and response of these species to the vaginal environment. Overall, this study provides a strong foundation for down-stream molecular work on *Candida* vaginal biofilms, showing that it is crucial to investigate species-specific virulence determinants for the vaginal environment in order to reveal new targets for the development of more efficient treatments for vulvovaginal candidiasis.

Disclosure statement

No potential conflict of interests was reported by the author(s).

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