Candida tropicalis biofilm and human epithelium invasion is highly influenced by environmental pH

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Abstract

Objective: The main goal of this study was to investigate the role of pH on *C. tropicalis* virulence determinants, namely the ability to form biofilms and to colonize/invade reconstituted human vaginal epithelia.

Methods: Biofilm formation was evaluated by enumeration of cultivable cells, total biomass quantification and structural analysis by scanning electron microscopy and confocal laser scanning microscopy. *C. tropicalis* human vaginal epithelium colonization and invasiveness were examined qualitatively by epifluorescence microscopy and quantitatively by a novel qPCR protocol for *Candida* quantification in tissues.

Results: The results revealed that environmental pH influences *C. tropicalis* biofilm formation as well as the colonization and potential to invade human epithelium with intensification at neutral and alkaline conditions compared to acidic conditions.

Conclusions: For the first time, we have demonstrated that *C. tropicalis* biofilm formation and invasion is highly influenced by environmental pH.

KEYWORDS: *Candida tropicalis*; biofilm; human vaginal epithelium; pH.

Introduction

In the last decades, the increase of candidiasis has been accompanied by an intensification of infections caused by non-*Candida albicans Candida* (NCAC) species, notably *Candida tropicalis* (Negri *et al.* 2010b; Silva *et al.* 2012)*.* Indeed*, C. tropicalis* has been described as able to colonize anatomically distinct sites, including the skin (Basu *et al.* 2003; Oksuz *et al.* 2007) and the gastrointestinal (Biasoli, Tosello and Magaró 2002), genitourinary (Basu *et al.* 2003) and respiratory tracts (Oksuz *et al.* 2007) and to cause superficial infections (Negri *et al.* 2012b; Quindós 2014). Moreover, *C. tropicalis* has been recognized as responsible for serious invasive candidiasis associated with high rates of morbidity and mortality (Negri *et al.* 2012a; Silva *et al.* 2012; Quindós 2014). The pathogenicity of *C. tropicalis* has been attributed to several virulence factors, such as the ability to evade host defenses, adherence, biofilm formation, secretion of hydrolytic enzymes and development of filamentous forms (Negri *et al.* 2010b; Sardi *et al.* 2013; Gonçalves *et al.* 2015).

The cell responses to environmental changes, such as pH, allow *Candida* species to take advantage of impaired immunity in debilitated patients and therefore facilitate the establishment of candidiasis (Schmidt *et al.* 2008; Vylkova *et al.* 2011; Pereira *et al.* 2015). The diverse niches that *C. tropicalis* inhabit vary prominently with respect to environmental pH. For instance, the pH of the oral environment varies significantly resulting from changes in diet, the metabolism of other constituents of the microbiota and salivary flow (Bensen *et al.* 2004). In the urinary tract, changes in the amount or type of acid produced is patient dependent with a urine pH ranging from 4.5 to 8 (Simon 2013). The vaginal tract is generally an acidic environment with a normal pH of 4.5, however there is an increase during the menstrual cycle and the vaginal tract becomes slightly alkaline (pH 7.5- 8) (Owen and Katz 1999; Davis 2003).

Adaptation to different pH has been shown to be critical for virulence in many commensals pathogens, specifically in *C. albicans* (Davis 2003; de Vasconcellos *et al.* 2014; Du and Hang 2015), but to our knowledge it was never evaluated for NCAC species in particular for *C. tropicalis*. Importantly, the effect of pH on expression of virulence factors is not known and could influence *C. tropicalis* pathogenicity. Thus, the present study aimed to investigate the role of pH on *C. tropicalis* virulence determinants, namely the ability to form biofilms and to colonize/invade a human vaginal epithelia.

Materials and methods

Organisms

A total of 7 clinical isolates of *C. tropicalis* recovered from different body sites, were used in this study. The isolates used were recovered from vaginal, urinary and oral tracts. *Candida tropicalis* strains 12 and 75 were recovered from the vaginal tract and obtained from the archive collection of the University of Maringá, Brazil. The oral isolates, AG1 and T2.2, were stock isolates from the Biofilm Group of the Center of Biological Engineering, originally isolated from Clinic of Dentistry, Congregados, Portugal. The urinary isolates, 519468 and 544123, were recovered from patients of the Hospital of S. Marcos, Braga, Portugal. The reference strain *C. tropicalis* ATCC 750 from the American Type Culture Collection was also examined. The identity of all isolates was confirmed using CHROMagar *Candida* (CHROMagar, Paris, France) and by PCR-based sequencing using specific primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (Williams *et al.* 1995).

Growth conditions

For each experiment, strains were subcultured on Sabouraud dextrose agar medium (SDA; Merck, Germany) for 48 h at 37 °C. Cells were then inoculated in Sabouraud dextrose broth (SDB; Merck, Germany) and incubated for 18 h at 37 °C under agitation at 120 rev/min. After incubation, the cells were harvested by centrifugation at 5000 x*g* for 10 min at 4 °C and washed twice with phosphate buffer saline (PBS 1%). The pellets were then resuspended in RPMI-1640 ("Roswell Park Memorial Institute"; Sigma-Aldrich, USA) and supplemented with MOPS 1M(3-(N-morpholino) propanesulfonic acid) to maintain the pH constant. In each experiment, the pH was controlled by the color of the RPMI medium (yellow in acid conditions, soft pink at neutral pH and strong pink at alkaline pH) and by measuring its value at the beginnings and end of each assay. The cell density was adjusted to 1×10^5 cells ml^{-1} using a Neubauer counting camera. The pH values tested were pH 3, 4, 7 and 8 and the adjustment was performed

by addition of sodium hydroxide to increase the pH values or by adding hydrochloric acid to decrease it.

Biofilm formation

In order to infer about the effect of pH on *C. tropicalis* biofilm formation standardized cell suspensions, 200 µl containing $1\text{x}10^5$ cells ml^{-1} in RPMI adjusted to different pH, were placed into wells of 96-well polystyrene microtiter plates (Orange Scientific, Belgium) and incubated at 37 °C on a shaker at 120 rev/min. After 24 h, 100 µl of RPMI medium was removed and an equal volume of fresh RPMI (adjusted to different pH) was added. The preparations were then incubated for a further 24 h. After this step, the medium was aspirated and non-adherent cells removed by washing the biofilms twice with phosphate buffer saline (PBS 1%). Then, the results were assessed using colony forming units (CFUs) enumeration and by crystal violet (CV) method. Three independent assays were performed, each with triplicate samples.

Biofilm characterization

Quantification of cultivable cells

The total number of cultivable cells in biofilms was determined by the enumeration of CFUs. Briefly the biofilms were washed, resuspended in PBS by repeated pipetting and subsequently the suspensions were vigorously vortexed for 2 min to disaggregate cells (Silva *et al.* 2010). Complete removals of the biofilms from the wells were confirmed by subsequent CV staining as described below. Serial 10-fold dilutions in PBS were performed and 10 µl of each dilution were plated on SDA at 37 °C an additional 24 h. The results were then expressed as the number of CFUs per unit of area (Log CFUs/cm^2).

Total biomass quantification

Total biomass biofilm quantification was assessed through CV staining methodology (Silva *et al.* 2009a). Thus, after washing, biofilms were fixed in the microtiter with 200 µl of methanol (100% v/v), which was removed after 15 min of contact. The microtiter

plates were allowed to dry at room temperature, and 200 μl of CV (1% v/v) was added to each well. After 5 min, the excess of CV was removed and the biofilms were gently washed with sterile ultra-pure water. Lastly, 200 μl of acetic acid (33% v/v) was added to release and dissolve the stain. The absorbance of the CV solution was read in triplicate in a microtiter plate reader at 570 nm. The results were presented as absorbance of the CV per unit of area (Abs/cm^2).

Quantification of filamentous forms

To evaluate the influence of pH on the development of *C. tropicalis* filaments, *C. tropicalis* were scraped from biofilms, observed using an epifluorescence microscope (Olympus BX51 coupled with a DP71 digital camera) (Olympus Portugal SA, Portugal) and the number of filaments quantified. All images were acquired using the Olympus Cell-B software and the length of the filaments was determined using the ImageJ Plug-in software.

Biofilm structure analyses

The structure of *C. tropicalis* biofilms and the morphology of biofilm cells were examined by scanning electron microscopy (SEM). For that, biofilms at pH 4, 7 and 8 were performed as describe above but in 24-well microtiter plates (Orange Scientific, Belgium). Thus, an inoculum of 1 ml of standardized yeast suspensions, 1×10^5 cells ml^{-1} in RPMI at different pH, was added to each well and biofilms were obtained as described previously. After biofilm formation, the samples were dehydrated with increasing concentrations of ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and then air dried for 20 min. Samples were kept in a desiccator until the base of the wells was removed for analysis. Previous to observation, the bases of the wells were mounted onto aluminum stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, USA).

Confocal laser scanning microscope (CLSM) was used to determine *C. tropicalis* biofilms thickness. For that, biofilms were dehydrated as described for SEM analysis and stained with Calcofluor white (Sigma-Aldrich, EUA), for 10 min at room temperature in the dark. The samples were stored in the dark until observation by

CLSM (Olympus BX61, Model FluoView 1000, Portugal). The excitation line 405 and the emission filters BA 430-470 (blue channel) were used, and images were acquired with the program FV10-ASW 4.2 (Olympus).

Biofilm matrix quantification

Biofilms for matrix quantification were also formed in 24-well polystyrene microtiter plates as described above. After 24 h, the medium was aspirated and an equal volume of fresh RPMI added and incubated for additional 24 h at 37 °C and 120 rpm. After 48 h, biofilm samples were scraped from the 24-well plates and resuspended with 1 ml ultra-pure water, sonicated (Utrasonic Processor, Cole-Parmer, IIIinois, USA) for 30 s at 30 W, and then the suspension was vortex for 30 s. The suspension was centrifuged at 3000 xg and 1 ml of supernatant used to determine the dry weight of each sample, filtered through a 0.45 μm nitrocellulose filter and the pellets were dried at 80 °C for 24 h until a constant dry biofilm weight was determined (Silva *et al.* 2009a).

Colonization and invasion of a reconstituted human vaginal epithelium (RHVE)

RHVE infection

Commercially obtained RHVE (SkinEthic Laboratories, France) inserts (0.5 cm^2) were placed in 12-well tissue culture plates and infected with 1 ml of *C. tropicalis* ATCC 750, T2.2 and 12 standardized suspensions (2 x 10^6 cells ml^{-1}) prepared in maintenance medium previously adjusted at pH 4, 7 and 8. As a negative control, 1 ml of maintenance medium adjusted to pH 4, 7 and 8 was added to a RHVE tissues preparation without *Candida* cells. Then, all infected tissues were incubated at 37 °C in a 5% CO_2 environment in saturated humidity for 12 h. After incubation, tissues were washed twice with 1 ml of PBS to remove non-adherent *C. tropicalis* cells. Tissues were then bisected, with one-half being used for fluorescence microscopy and the other for quantification of *Candida* cells infecting RHVE by quantitative real-time PCR (qPCR). Two different tissue samples in two independent infections were used for each condition.

Microscopic observation

The tissues for microscopic analysis were fixed in 4% (w/v) paraformaldehyde and stored at 4 °C for 24h until histological processing. Then, the tissues were dehydrated, paraffin wax-embedded, cut into 5 µm sections (HM 325, Microm, Germany) and finally placed on microscope slides (Histobond, UK). Before staining, the sections were dewaxed and hydrated. Sections were immersed in xylol (Fisher Chemical, U.K.) for 15 min, hydrated with decreasing concentration of ethanol (Panreac, Spain) (100% and 50% for 5 min each time) and distilled water for 5 min. Tissues were allowed to air dry and subjected to the staining. Concanavalin A ALEX FLUOR 488 (Molecular Probes-Invitrogen, USA) and 4',6-diamidino-2-phenylindole (DAPI- Molecular Probes-Invitrogen, USA) dyes were used to ascertain the extent of invasion of the RHVE by *C. tropicalis* strains, at different pH values. For that, tissue sections on microscope slides were overlaid with one drop of concanavalin A dye in the dark for 10 min at room temperature. For cell nucleus detection, human cells were stained with DAPI for additional 10 min at room temperature. After this, the preparation was washed with water, allowed to air dry and mounted with one drop of mounting oil (Merk, Germany). The slides were stored in the dark for a maximum of 12 h before microscopy visualization. The microscopy results were analyzed using an epifluorescence microscope Olympus BX51 coupled with a DP72 digital camera and equipped with 3 sets of filters (DAPI - 365-370/421, FITC - 470-490/516 e TRITC - 530-550/591)(Olympus Portugal SA, Portugal). All images were acquired using the Olympus Cell-B software.

Quantification of Candida cells in RHVE

For DNA extraction, half sections of 5 µm of infected tissues were cut and placed in sterile 1.5 ml microcentrifuge tubes (Eppendorf AG, Germany) with approximately 300 µl of glass beads (0.5 mm diameter − Sigma-Aldrich, EUA) and 600 µl of sorbitol buffer (GRISP, Portugal). This final mix was homogenized three times for 60 s, using a Mini-Beadbeater-8 (Stratech Scientific, UK) and the supernatant was carefully placed in another sterile microcentrifuge tube. Subsequently, the DNA extraction was performed using a commercial DNA extraction kit (QIAamp® DNA FFPE Tissue, Qiagen, UK) in accordance with the manufacturer's protocol. After extraction, the DNA from each

experimental condition was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA).

Quantitative real-time PCR (qPCR)

Candida tropicalis cells of each strain in the infected RHVE were quantified using real-time PCR employing a CF X96 Real-Time PCR System (Bio-rad, USA). Each reaction mixture consisted of 10 µl of working concentration of SsoFast EvaGreen Supermix (Bio-rad, USA), 0.2 µl of each primer (50 µM) designed previously (Actin: forward-GACCGAAGCTCCAATGAATC and reverse-AATTGGGACAACGTGGGTAA) and 4 µl of DNA, in a final reaction volume of 20 µl. Negative controls were performed using a reaction mixture with nuclease free water (Cleaver Scientific Ltd, UK) substituting for the template DNA. PCR cycling conditions consisted of an initial denaturation step at 98 °C for 2 min, followed by 40 cycles of denaturation at 98 °C for 5 s and primer annealing at 60 °C for 5 s. In each cycle, a dissociation stage of 60 °C was run to generate a melting curve for confirmation of the specificity of the amplification product. Calibration curves (Ct vs. log cells) for each of the *C. tropicalis* isolates were constructed using the same PCR protocol as described above. For these, serial dilutions of *C. tropicalis* cells were prepared using an improved Neubauer haemocytometer (Marienfeld, Germany) and the DNA for PCR analysis extracted from the cell pellet using the DNA extraction kit (QIAamp® DNA FFPE Tissue, Qiagen, UK) with some modifications.

Lactate dehydrogenase (LDH) activity

The extracellular activity of LDH released from RHVE cells was monitored as an indicator of tissue damage. LDH released in the maintenance medium of the control RHVE which was devoid of *Candida*, as well as infected RHVE was measured at 12 h using the CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega, USA). LDH activity from *Candida* cells in a planktonic control (prepared as described for the infection model but devoid of RHVE) was subtracted from the LDH activity of the

tissues infected with the different *C. tropicalis* clinical isolates. The LDH released during infection with *C. tropicalis* cells was then expressed as relative LDH activity to the untreated control tissue. LDH activity was analysed spectrophotometrically (FLUOstar OPTIMA; BMG Labtech, Germany) by measuring the NADH disappearance rate at 544 nm excitation and 590 nm emission during the LDH-catalyzed conversion of pyruvate to lactate. All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, CA, USA). The results obtained in the studies were compared using analysis of variance (ANOVA) by applying Tukey multiple comparison test. All tests were performed with a 95% confidence level.

Results and Discussion

Candida tropicalis predisposition for dissemination and the high mortality associated with its infections might be strongly related to the potential of virulence factors exhibited by this species and its high capacity of adaptation to the diverse human niches (Negri *et al.* 2012b). The change of ambient pH is one of the major challenges often encountered by this pathogen. *Candida tropicalis* encounters a wide range of pH stresses during its commensal and pathogenic lifestyles (Du and Hang 2015), however there is a lack of knowledge about its influence on *C. tropicalis* pathogenicity. Thus, the main goal of this study was to investigate the role of pH on *C. tropicalis* biofilm formation and in its ability to colonize human vaginal epithelia.

Influence of pH on Candida tropicalis biofilm formation

The ability to form biofilms of the *C. tropicalis* reference strain ATCC 750 and of six *C. tropicalis* isolates recovered from oral (AG1 and T2.2), vaginal (12 and 75) and urinary (5144123 and 519468) tracts was evaluated at pH 3, 4, 7 and 8. The results were studied in terms of the number of cultivable cells (log CFUs/cm^2) and total biomass (Abs/cm^2) (Fig. 1). *C. tropicalis* strains were able to form biofilms, but with some

differences according to the strain and the pH. These results are in line with the results published by Silva *et al*. (2009) and Negri *et al*. (2010) that showed high diversity concerning the capability of the *C. tropicalis* strains to form biofilms. It is important to stress that, in general, the ability to form biofilms varied with the environmental pH being more pronounced at neutral and alkaline pH (Fig. 1-I.). In fact, the number of cultivable cells present in the biofilm increased with the pH and the differences were more significant at pH 8, namely in the case of the strains 519468 and 12 ($p<0.05$) (Fig.1-II.). Importantly, it was also noticed that, generally, *C. tropicalis* had an increase in total biomass at pH 7 and 8 compared to pH 3 and 4, wherein biofilms formed at pH 8 presented the highest levels of biomass. Especially at pH 7 and 8 the increase was superior ($p<0.05$ for *C. tropicalis* 75 and 519468) to what we observed in cultivability assays (no significant differences for *C. tropicalis* 75 and for *C. tropicalis* 519468 ($p<0.05$)) (Fig.1). In this work we demonstrated that *C. tropicalis* fully adapts to pH variations being able to grow at acidic, neutral and moderately basic pH. This is an interesting result, especially when related to the potential virulence of this species, whose infections occur in niches with different values of pH (Owen and Katz 1999; Davis 2003). Moreover, it is known that biofilm is a lifestyle that confers to the microorganisms an ecological advantage, aiding survival as commensals and pathogens of humans by allowing them to evade host immune mechanisms, resisting antifungal treatment and even as a way to adapt to environmental stress conditions (Silva *et al*. 2009a; Mahami 2011).

Another important virulence factor of *Candida* species is the ability to develop filamentous forms (Davis 2003; Schmidt *et al.* 2008; Silva *et al.* 2011, 2012; Negri *et al.* 2012b). Therefore, the *C. tropicalis* reference strain ATCC 750, the oral isolate AG1 and T 2.2, the vaginal isolate 12 and 75 and the urinary isolate 5144123 and 519468 were chosen for the SEM analysis and matrix quantification. SEM analysis was used to examine the effect of the pH on *C. tropicalis* biofilm structure and morphological characteristics of biofilm cells. *C. tropicalis* biofilms included different morphotypes, in a strain and pH dependent manner (Fig. 2-I.). At pH 4 biofilms of *C. tropicalis* strains were composed of both yeast and pseudohypha, with exception of strain 75 that exhibited only yeast morphology (Fig. 2-I.). Importantly, for all strains an increase in pseudohyphal cells with medium alkalization was observed. Additionally, it was possible to observe that *C. tropicalis* biofilm structures were affected strongly by the pH tested. At pH 4 and pH 7, *C. tropicalis* biofilms presented as a discontinuous monolayer

in contrast to biofilms formed at pH 8 that displayed a multilayer and compact biofilm covering the entire surface (Fig. 2-I.). Interestingly, at pH 7, *C. tropicalis 75* already had a similar structure to that observed at pH 8 (Fig. 2.1). SEM images also revealed the presence of matrix at pH 7 (*C. tropicalis 75*) and at pH 8 for all strains (see asterisks), compared to pH 4 (Fig. 2-I.). Epifluorescence microscopy (Fig. 3-I.) and SEM images (Fig. 2-I.) revealed that alkaline conditions induce *C. tropicalis* filamentation. These results are in agreement with those of other authors, who reported adaptation to environmental pH by *C. albicans* and its relation to pathogenesis (Davis 2003; Schmidt *et al.* 2008; Fisher *et al.* 2011; Vylkova *et al.* 2011; Ene *et al.* 2012). Several authors have suggested that the presence of filaments may have importance in the structural integrity of multilayered biofilms (Ramage, Saville and Thomas 2005; Silva *et al.* 2009a, 2012), which might explain the compact (Fig. 2-I.) and thick (Fig. 2-II.) biofilms obtained at pH 8.

One of the important characteristics of fungal biofilms is the presence of the extracellular matrix. There is a general consensus that the biofilm matrix acts as a protective barrier against a wide range of environmental conditions (Al-Fattani and Douglas 2004; Silva *et al.* 2009a). In order to analyze this, CLSM was used to assess biofilm structure and to determine the thickness of biofilms (Fig. 2-II.). The CLSM observations corroborated the SEM images demonstrating an intensification of the biofilms thickness with the increment of pH (Fig. 2-I. and 2-II.). Indeed, *C. tropicalis* ATCC 750 presented an increase of 8 μm to 13 μm and to 14 μm (Fig. 2-II. - A, B and C) and *C. tropicalis* 519468 from 8 μm to 11 μm and to 16 μm (Fig. 2-II. - A, B and C) respectively for pH 4, 7 and 8. *Candida tropicalis* biofilm matrices were also quantified (Fig. 2-III.) and the results showed, in general, a significant increase in the matrix dry weight with the pH, mainly at pH 7 and 8 comparatively to pH 4. In fact, *C. tropicalis* biofilms have a greater amount of matrices at neutral and alkaline compared to acidic pH (Fig. 2-III.). This result corroborated the increase in biofilm biomass detected in Fig. 1-I. The percentage of biofilm cells with filaments was quantified for pH 4, 7 and 8 (Fig. 3). In general, it was possible to observe that the percentage of filamentation increased significantly at pH 8 ($p<0.05$) compared to pH 4 and 7, with exception of *C. tropicalis* AG1 ($p<0.05$). Nevertheless, it is important to point out that the AG1 strain presented a similar percentage of filamentation at all pH tested (Fig. 3). In the case of *C. tropicalis* 75, the data showed that this strain was unable to develop filamentous forms at pH 4 but was able to develop filaments at pH 7 and 8 (Fig. 3). However, one of the

most important results obtained was the increase in length of filaments with the increasing pH (Fig. 3-II.). Indeed, *C. tropicalis* ATCC 750 presented an increase of the filaments' length from 0 μm to 101 μm and to 140 μm and *C. tropicalis* 75 from 64 μm to 70 μm and to 174 μm for pH 4, 7 and 8, respectively. Although these clinical isolates, have been recovered from different host niches, they showed similar behavior when exposed to different pH.

Influence of pH on Candida tropicalis RHVE colonization and invasion

In order to deepen the knowledge about the influence of pH on *C. tropicalis* pathogenicity, a commercially available reconstituted human vaginal epithelium was used and infected at the different pH. The colonization and invasion of RHVE was examined after 12 h of infection with *C. tropicalis* ATCC 750 (reference strain), T2.2 (oral isolate) and 12 (vaginal isolate) at pH 4, 7 and 8 (Fig. 4-I.). The results showed that all *C. tropicalis* strains were able to colonize the RHVE as reported by Silva *et al.* (2009b), at every pH tested. Furthermore, this work revealed that the extent of colonization and invasion was noticeably pH and strain dependent. Microscopic images showed an increase in the level of colonization and invasion with the increase of pH from 4 to 7 and to 8. Moreover, *C. tropicalis* clinical isolates presented diverse cell morphologies on the RHVE surface at various pH. At pH 4, *C. tropicalis* ATCC 750 and 12, were unable to invade the RHVE exhibiting only a network of yeasts in the superficial layer, while *C. tropicalis* T2.2 presented some pseudohyphal enabling invasion of the first layer (Figure 4.1 - A1, C1 and B1). At pH 7 and 8, in all *C. tropicalis* isolates, yeasts and pseudohyphal forms were observed together with an increase of invasion (Fig. 4-I. - A2/A3 and B2/B3 e C2/C3). Interestingly, despite observing some increase in invasion at pH 7 (Fig. 4-I. - A2, B2 and C2) the highest invasiveness was observed at pH 8 with fungal filaments totally penetrating the RHVE layers (Fig. 4-I. - A3, B3 and C3). Polymorphism and hyphal growth are factors, previously recognized in various studies, able to improve and facilitate RHVE colonization and invasion (Silva *et al.* 2009b; Alves *et al.* 2014a, 2014b). In the case of *C. tropicalis*, the invasiveness also increased with the increment of the filaments formed at neutral and basic pH. Furthermore, *C. tropicalis* filaments invasion reinforces the damage of the epithelium (Fig. 4-I. and 4-III.). Quantitative PCR was used to quantify the number of the *C. tropicalis* cells infecting RHVE. The results showed that *C.*

tropicalis ATCC 750 presented an increment of cells infecting the tissues coupled with the increase of pH from 4 to 8, corroborating the microscopic observations (represented in Fig. 4-I. and Fig. 2). However, concerning *C. tropicalis* T2.2 and 12, the number of cells was similar for all pH values. In order to determine the extent of RHVE damage induced by *C. tropicalis* cells, the levels of LDH activity were measured. Co-culture of the tissue with each *C. tropicalis* isolates caused an increased damaging effect at pH 7 and 8 comparatively to pH 4 ($p<0.05$), with exception of *C. tropicalis* ATCC 750 that demonstrated similar damage between pH 4 and pH 7 ($p>0.05$) (Fig. 4-III.). The pH tested did not influence the basal levels of the LDH released from uninfected epithelium (data not shown). It was been demonstrated by other investigators that for *C. tropicalis* (Okawa *et al*. 2008; Silva *et al*. 2011) aspartyl proteases (Saps) are not required for invasion and damage to reconstituted human oral epithelium. Additionally, Yu *et al.* 2016, demonstrated recently a significant correlation between *C. tropicalis* adhesion to epithelial cells, the expression of three Agglutinin-like sequences (*ALST2-3*) and cell damage. Thus, *C. tropicalis ALST2-3* can be one of the factors involved in the RHVE damage observed in this work.

Additionally, this study revealed that pH does not influence epithelial morphology and structure (Fig. 4-I.). It must be emphasized that, despite the results observed concerning invasion of vaginal cells, further investigations with other human epithelia (oral and/or gastrointestinal) and *in vivo* studies are required to confirm and validate these results.

In summary, the present study demonstrated that *C. tropicalis* biofilm formation and vaginal epithelium invasion is highly influenced by environmental pH. Furthermore, we showed an increase in biofilm formation, as well as in colonization and invasion of a human vaginal epithelium coupled with filamentation at neutral and alkaline conditions compared to that observed in acidic conditions. If these effects can be extrapolated to the *in vivo* situation, then pH levels may play an important role in the progression of *C. tropicalis* infections. Through elucidating such inherent differences, it might be possible to identify and specifically combat strains adapted for infection at particular body sites controlling the environmental pH.

Acknowledgements

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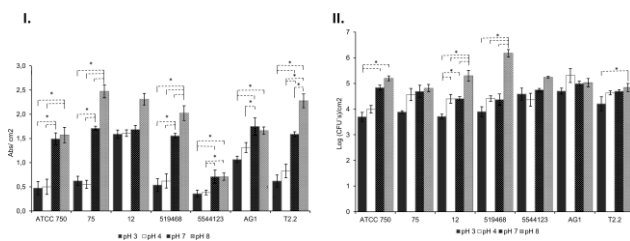
Fig. 1- Biofilm quantification analyses. (I.) Absorbance values of Crystal Violet solutions (Abs/cm^2) and (II.) Cell cultivability (Log (CFUs)/cm^2) of *Candida tropicalis* biofilms formed during 48h in RPMI at different pH (3, 4, 7 and 8). Error bars represent standard deviation. *p<0.05 (one-way ANOVE, Turkey multiple-comparisons test. The experiments were performed in three independent assays, each in triplicate.

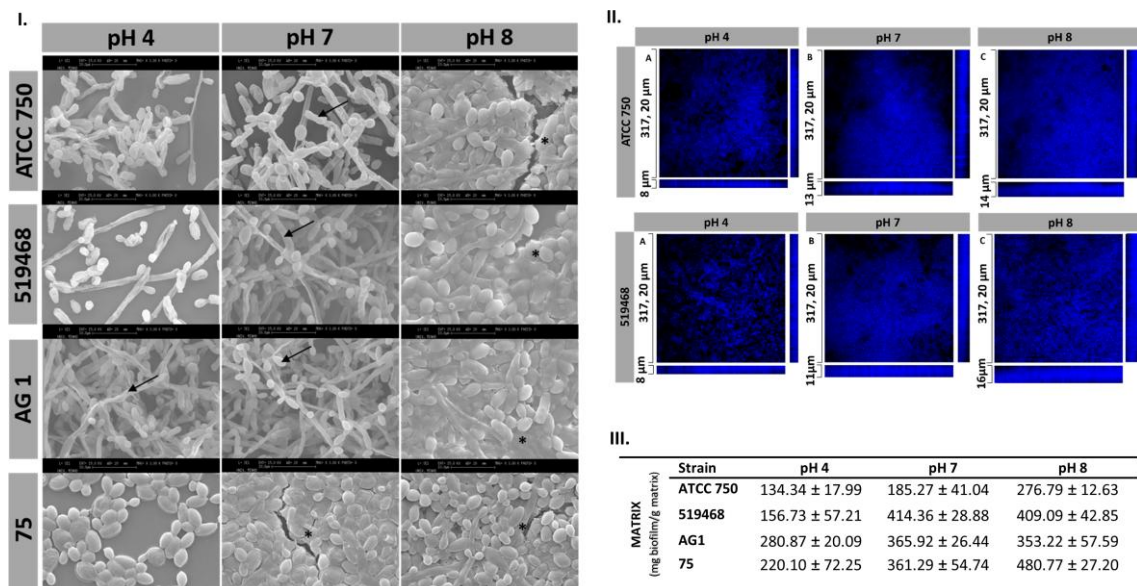|	Strain	pH 4	pH 7	pH 8
MATRIX (mg biofilm/g matrix)	ATCC 750	134.34 ± 17.99	185.27 ± 41.04	276.79 ± 12.63
	519468	156.73 ± 57.21	414.36 ± 28.88	409.09 ± 42.85
	AG1	280.87 ± 20.09	365.92 ± 26.44	353.22 ± 57.59
	75	220.10 ± 72.25	361.29 ± 54.74	480.77 ± 27.20

Fig. 2- Biofilm structure analyses. (I.) Scanning electron microscopy, (II.) Confocal laser scanning microscopy images and (III.) Biofilm matrices quantities (mg biofilm/g matrix) of *Candida tropicalis* biofilms formed during 48h in RPMI at different pH (4, 7 and 8). The SEM scale image corresponds to 20 μm. Magnification X 1000. Arrows indicates the presence of pseudohyphal form and asterisk presence of matrix. The values are means ± standard deviations were generated in three independent experiments, each performed in triplicate.
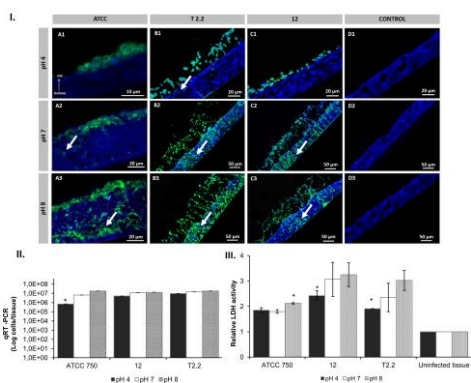
Fig. 3- Quantification of filamentous forms. (I.) Percentage of filament cells (%) and (II.) Optical micrograph of *Candida tropicalis* biofilms cells formed during 48h in RPMI at different pH (4, 7 and 8). Error bars represent standard deviation. * Statistical difference obtained when compared with the different pH tested ($p<0.05$). The scale in the images corresponds to 50 μm. The experiments were performed in three independent assays, each in triplicate.
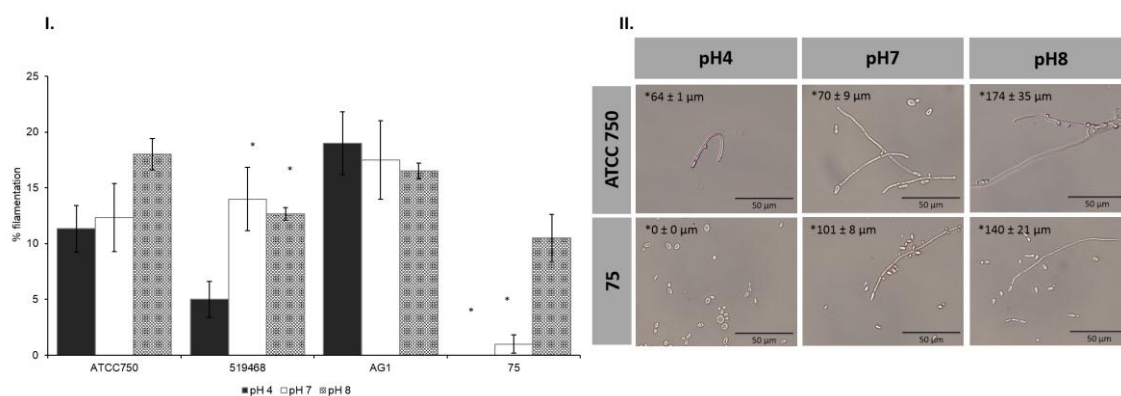

Fig. 4- Reconstituted human vaginal epithelium invasion and colonization analyses. (I.) Epifluorescence microscopy of reconstituted human epithelium infected with *Candida tropicalis* during 12 h at different pH (4, 7 and 8). The right column represents the uninfected epithelium. Blue-fluorescence corresponds to reconstituted human epithelium and green-fluorescence corresponded to *Candida tropicalis* cells. The xy-axis on image A1 indicates tissues orientation. Arrows indicate invasion of *Candida tropicalis* cells. (II.) qPCR quantification of *Candida tropicalis* infecting the reconstituted human epithelium (Log cells/tissue) (III.) Relative lactate dehydrogenase (LDH) activity. Error bars represent standard deviation. * Statistical difference obtained when compared with the different pH tested ($p<0.05$). The experiments were performed in two independent assays, each in duplicate.