TÉCNICO LISBOA

Master in Biotechnology

Effect of the presence of acetic acid and lactic acid at low pH in physiology and virulence of *Candida albicans* and Candida glabrata: emphasis on the role played by genes of the CgHaa1-regulon

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Abstract: In order to successfully colonize the vaginal niche *C. glabrata* has to face multiple environmental insults, which include, among others the presence of lactic and acetic acids that are produced by the commensal bacterial microflora. The acidic pH (~4) of the vaginal tract potentiates a toxic effect of these carboxylic acids, this being thought to contribute for the restrain of the progress of infections caused by pathogens, including by *Candida* spp. In this work it was examined how the presence of concentrations of acetic and lactic acids similar to those found in the vaginal tract affect growth *of C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314 at acidic pHs. Under the conditions used lactic acid had no inhibitory effect, while acetic acid significantly inhibited growth of the two strains, this effect being more prominent for *C. albicans* SC5314. Furthermore, the presence of acetic and lactic acids at low pH was also found to modulate tolerance of the two strains to azoles and also adherence of the strains to polystyrene. A second aspect that was also developed in this work was the functional analysis of the CgHaa1-pathway, recently shown to be involved in C. glabrata response to acetic acid stress. In specific it was demonstrated that a set of adhesins found to be regulated by this transcription factor (*Ad1, Ad2, Ad3 and Ad4*) are required for adhesion and subsequent colonization of vaginal epithelial cells in the presence of acetic acid. Furthermore, it was also shown that *CgHaa1* is required for *C. glabrata* virulence against the infection models *C. elegans* and *G. mellonella* this extending the biological function of this regulatory system beyond response to acetic acid.

Introduction

Vulvovaginal candidiasis (VVC) is the infection of the vulva and/or the vagina caused by species of the *Candida* genus. It is the second most common cause of vaginal infections (Nagashima et al. 2015) being estimated to affect 70-75% of women at least once in their lifetime, 40-50% of them experiencing at least one recurrence (Rosa et al. 2013). About 13% to 19% of VVC patients have repeated occurrences of this infection, a condition known as recurrent vulvovaginal candidiasis (RVVC) (Kabir et al. 2012; Foxman et al. 2013). In certain conditions the commensal Candida populations may become pathogenic and induce infections which can range from mild superficial infections (in the oral or in the vaginal tract) to large disseminated mycoses in which the yeasts cross the bloodstream and may colonize any major organ (Wong et al. 2014). The majority of VVC and RVVC cases are caused by *C albicans*; however, the incidence of non-*Candida albicans* species of *Candida* (NCAC) is increasing, specially in what concerns to the infections caused by *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. parapsilosis* (Mintz & Martens 2013).

To successfully colonize each niche *Candida* spp. have to face numerous environmental insults including alterations in pH, in nutrient availability (Merhej et al. 2016), the activity of the host immune system (Nevitt & Thiele 2011) and also the presence of a microbiota that competes for nutrients and for adhesion sites (Hasan et al. 2009). Despite this, metagenomic analyses undertaken with different female populations have been showing that *Lactobacilii* species are always predominant in the vaginal microflora (Doerflinger et al. 2014). The metabolic activity of commensal bacteria often results in the production of carboxylic organic acids such as acetic acid, lactic acid, butyric acid, among others (Aldunate et al. 2013; Ilkit & Guzel 2011; Aldunate et al. 2015; Owen & Katz 1999). The production of these acids are behind the acidification of the vaginal pH to 3.5-4.5, values that inhibit growth of most pathogenic bacteria (Boskey et al. 1999; Jeavons 2003). Consistently, significant amounts of lactic and acetic acids (55–111 mM and 1–100 mM, respectively) are found present in the vaginal tract (Aldunate et al. 2013; Ilkit & Guzel 2011; Aldunate et al. 2015; Bernardo et al. 2016).

This thesis is focused on the study of the effects exerted by acetic and lactic acid at acidic pHs (similar to those found in the vaginal tract) in the physiology and also in some virulence traits of *C. albicans* and *C. glabrata*. In specific it

was assessed the tolerance levels of two lab strains of *C. glabrata* (the reference strain CBS138 and the vaginal strain BG2), and of one lab strain of *C. albicans* (SC5314) to acetic and lactic acids in concentrations and pHs similar to those described to exist in the vaginal tract. The purpose of this was to perform a comparative analysis since the studies undertaken until so far have examined this issue in one of these species and using different experimental setups (e.g. different growth media, pH, among others). Considering that the constant presence of these organic acids in the vaginal environment, it was also examined how they can modulate resistance of *C. albicans* and *C. glabrata* to antifungals currently used to treat vaginal candidiasis including clotrimazole, fluconazole, miconazole and tioconazole. On a different, but complimentary perspective, the recently performed functional analysis of the CgHaa1 regulon, essential for tolerance to acetic acid in *C. glabrata*, further was complemented by examining the role played by the CgHaa1-regulated adhesins Ad1, Ad2, Ad3 and Ad4 in adhesion of C. glabrata to biotic and abiotic surfaces during growth in the presence of acetic acid at a low pH. An eventual role of CgHaa1 and of these AD1, AD2, Ad3 and Ad4 in determining virulence of *C. glabrata* against the infection models *G. mellonela* and *C. elegans* was also scrutinized.

Materials and methods

Strains, growth media and cultivation conditions.

The *C. glabrata* strains kindly provided by Professor Hiroji Chibana from Chiba University in Japan. The *Candida* strains used were batch-cultured at 30°C, with orbital agitation (250 rpm) in minimal medium (MM) containing per liter: 20 g glucose; 2.7 g $(NH_4)_2SO_4$; 1.7 g yeast nitrogen base without amino acids, in YPD (10 g/L, yeast extract, 20 g/L peptone and 20 g/L glucose), in RPMI (10,4 g of RPMI 34,5 g of MOPS (Sigma) and 18 g of glucose) In the case of RPMI 0,2% glucose, no glucose was added. BHI medium (37g/L of powder - Sigma). When required the pH of the different growth media was adjusted using HCl and NaOH. All media were prepared in deionized water and sterilized by autoclaving for 15 minutes at 121°C and 1 atm, except RPMI that was sterilized by filtration. Solid media were obtained by supplementing the corresponding liquid growth medium with 20 g (per liter) of agar (Iberagar).

Preparation of acetic and lactic acids stock solutions and antifungals stock solutions

A 2M stock solution of acetic and lactic acids was prepared using deionized water as a solvent. The pH of these stock solutions was adjusted to pH 4.0 using NaOH 10 M and/or HCl. After pH adjustment the solutions were sterilized by filtration using a sterile and endotoxin free 0.2 µm PES filter – PuradiscTM 25 mm. The stock solutions of the antifungals, clotrimazole, miconazole, fluconazole and tioconazole were prepared from the powder and using DMSO (Dimethyl sulfoxide, Sigma) as the solvent. All antifungals were purchased from Sigma. The stocks were prepared at the following concentrations: fluconazole 10 mg/mL, tioconazole 1 mg/mL; clotrimazole 20 mg/mL and miconazole 1 mg/mL.

Susceptibility assays undertaken in the presence of acetic and lactic acids and azoles

The susceptibility of *C. albicans* SC5314, *C. glabrata* CBS138 and *C. glabrata* BG2 to acetic and lactic acids was tested in RPMI containing 0,2% of glucose and in MM growth media containing 0,2 or 1% glucose. In all cases the cultivation assays were performed in 96-multiwell microplates. Five concentrations of each acid were tested: for acetic acid 0.4, 4, 30, 45 and 75 mM, while for lactic acid it was used 80, 100, 120, 140, 160 mM.. The media and the organic acids concentrations were adjusted at 4 pHs: 4.5, 4, 3.5 and 3 using HCl as the acidulant..

Susceptibility of *C. glabrata CBS138, C. glabrata* BG2 and *C. albicans SC5314* to clotrimazole, fluconazole, miconazole and tioconazole in the presence of lactic or acetic acids was performed using an experimental setup similar to the one described above. The concentrations of antifungals used were: 1, 5, 7.5 and 10 mg/L for clotrimazol; 30, 60, 64 and 128 mg/L for fluconazole; 0.05, 0.2 and 0.4 mg/L for Miconazol and 0.1, 0.3, 0.45 and 0.6 mg/L for Tioconazol. In all cases the stock solution of the antifungal was adjusted at pH 4.

The cell suspensions (set an $OD_{600\,nm}$ of 0.2) used as inocula were obtained by diluting a culture in mid-exponential phase (OD_{600nm} of ~0.8) and in each well was added 50 µL of each cellular suspension The plates were incubated at 30ºC (using an agitation of 20rpm or 200rpm) for 24h and growth was accompanied based on the increase in OD_{600nm}.

Adhesion to polystyrene

For this cells of the different species were cultivated until mid-exponential phase (OD_{600nm} approximately 0.8), diluted to an initial OD_{600nm} of 0.2 and finally inoculated in the 96-multiwell plate yielding an initial OD of 0.1. After 6 h or 24h of cultivation non-adherent cells were removed from each well and the adhered cells were washed twice with 200 µL sterile PBS. Afterwards, 200 µL of crystal violet was added and after 15 minutes the excess of CV was removed and all wells washed three times with deionized water. In the end 200 µL of ethanol 96% was added to release the CV bound to the cells. The absorbance was measured at 590 nm and it was taken as a measurement of the number of adhered cells (Peeters et al. 2008).

Minimum inhibitory concentration (MIC)

The comparison of susceptibility to azoles was undertaken using the method recommended by EUCAST (http://www.eucast.org). The azoles tested were fluconazole (0.125-64 mg/L), miconazole (0.06-32 mg/L), Tioconazole (0.03-16 mg/L) and clotrimazole (0.06-32 mg/L). *C. glabrata* KCHr606 cells and the derived deletion mutants ΔAD1, ΔAD2, ΔAd3 and ΔAd4 were cultivated, at 30ºC and 250 rpm, in 5mL of YPD during 18h**.** After that time the inoculum was

suspended by vigorous shaking on vortex mixer and an appropriate volume was transferred to a new tube to prepare an aqueous cell suspension, having an OD_{600nm} of 0.025 corresponding to around 1.25x105 CFU/mL which is within the range of 0.5x105 – 2.5x105 CFU/mL recommended by the EUCAST protocol. 100 µL of this cell suspension was then inoculated in a 96-multiwell plate containing 98 µL of RPMI 2% glucose and 2 µL of the different antifungals tested. When the assays were done with 30 mM of acetic acid the OD_{600nm} of the cellular suspension were set in 0.05, and added 50 µL of this cell suspension was then inoculated in a 96-multiwell plate.The assays were performed at pH 4. The microplates were incubated without agitation at 37ºC during 24h after which the absorbance at 590nm was measured.

Adherence of *C. glabrata* and to reconstituted vaginal human epithelium

The assays performed to study the effect of acetic acid in infection of vaginal epithelium were performed by Dr. Sónia Silva from Centro de Engenharia Biológica da Universidade do Minho, and are fully described in Bernardo *et al*. 2016.

C. glabrata* virulence against *C. elegans

C. glabrata against the infection model *C. elegans* was performed using strain glp-4 which is unable to produce gonads or progeny at 25°C (Pukkila-worley et al. 2009). The worms were maintained and propagated in NGM2 growth media using *E. coli* OP1 as the only source of food. The methodology used in this work was based on a previously published experimental setting (Pukkila-worley et al. 2009; Pukkila-worley et al. 2011) Briefly, C. glabrata KUE100 cells and the derived deletion mutants ΔHaa1, ΔAD1, ΔAD2, ΔAd3 and ΔAd4 used to infect C. elegans were cultivated over-night in 5 mL of YPD. 10µL of this culture were spread on solid brain heart infusion agar medium (4 mL plates) containing 45 mg of kanamycin/ml and let to grow at 30oC for 24h. On the next day the plates were scrapped with a sterile loop to obtain a uniform layer of yeast cells. Afterwards, an average of 15-30 worms were added to the scrapped plates in such a way that C. glabrata cells were the only food available for the worms. The assays were performed in triplicate using 5 plates for each strain.

C. glabrata virulence against *Galleria mellonella*

Maintenance of *Galleria mellonella* larvae was performed as described in (Mil-Homens et al. 2016). Briefly, the larvae were reared in an insectarium on a pollen grains and bee wax diet at 25ºC in darkness. Last instar larvae weighing 250±25mg were used for the survival experiments. *C. glabrata* KUE100 cells or the derived deletion mutants ΔAD1, ΔAD2, ΔAd3 and ΔAd4 were cultivated overnight in 5 mL of YPD at 30ºC. After this, an appropriate volume of this culture was taken to prepare a diluted cell suspension (using PBS as the solvent) having approximately $1x10^7$ cells per volume of injection (4 µL). A micrometer was adapted to control the volume of a microsyringe and each larvae was infected with 3,5 µL of the each cell in the hindmost left proleg, previously sanitized with 70% (V/V) ethanol. After injection, larvae were placed in Petri dishes and stored in the dark at 37ºC. Control larvae were injected with sterile PBS (pH 7.4). For each strain we used 10 larvae were followed to assess survival in a period of 3 days. Caterpillars were considered dead when they displayed no movement in response to touch

Results

***C. glabrata* and *C. albicans* susceptability to lactic and acetic acids at a low pH**

The assessment of the potential inhibitory effect of lactic or acetic acids on growth of *C. glabrata* and *C. albicans* was tested in MM medium either having 1% or 0.2%, corresponding, respectively, to a condition where glucose is non-limiting or limiting. Two strains of *C. glabrata* were used for this: strain CBS138, which has an intestinal origin, and the BG2 strain, which is a vaginal isolate (Koszul et al. 2003; Nash et al. 2016). In the case of *C. albicans* it was used the reference strain SC5314, which was collected from the bloodstream of an infected patient. The pH of the MM media was adjusted to 4.5, 4, 3.5 and pH 3. These acidic pHs were selected because they are representative of the pH range described to occur in the vaginal tract (Boskey et al. 1999; Jeavons 2003) and also because they are close to, or even below, the pKa values of acetic (4.76) and lactic acids (3,86), favoring the predominance of the toxic undissociated form. A range of 0.4-75 mM of acetic and 80-160 mM of lactic acid were tested, these also being representative of the concentrations described to exist in the vaginal tract (Owen & Katz 1999).

In what concerns to susceptibility of the different strains to acetic acid at pH 4.5 (the highest pH tested) the results obtained clearly demonstrated that *C. albicans* SC5314 is highly susceptible to this organic acid, while the two *C. glabrata* strains were found to be more resilient (Figure 1). The reduction of glucose concentration from 1 to 0.2% led to decrease in growth of the strains, although the pattern of tolerance observable is the same with C. albicans SC5314 being the more sensitive followed by *C. glabrata* CBS138 and *C. glabrata* BG2. In the glucose-limited medium, the *C. glabrata* CBS138 strain was found to be less tolerant to acetic acid than the *C. glabrata* BG2 strain, this also being reported in previous studies and being attributed to the evolution of efficient adaptive responses to cope with organic acids at low pH of vaginal strains (Gregori et al. 2007). It is interesting to observe that this effect was only detectable when the concentration of glucose available was scarce. Expectedly, the reduction in pH of the MM medium potentiated the toxic effect of acetic acid and, consequently, exacerbated the susceptibility exhibited by the strains this effect being more prominent for *C. albicans* SC5314.

Cultivation of the strains in the presence of lactic acid resulted in a small enhancement in growth. It is possible that this beneficial effect could result from the reported ability of *C. glabrata* and *C. albicans* to use lactic acid as a carbon

source, even if glucose is present in the growth medium (Childers et al. 2016). Under aerobic conditions, the exposure of the three *Candida* strains to lactic acid under microaerophilic conditions (20 rpm) also did not resulted in growth inhibition, independently of the concentration of acid used or of the medium pH.

The results were obtained in fully aerobic conditions (using 200 rpm orbital agitation) and since in the vaginal environment the concentration of oxygen is variable we have decided to perform the overall susceptibility analysis under microaerophilic conditions. For that the cells were cultivated under the same experimental setting with the difference that the orbital agitation of the culture was reduced to 20 rpm. In general, the results obtained, had no significant difference in the results obtained under aerobic or microaerophilic conditions indicating that the availability of oxygen does not seem to play a critical role in modulation of the tolerance of the two strains to acetic or lactic acids. *Optical density at 600$_{nm}$ of* C. glabrata *CBS138,* C. glabrata *BG2 and* C. albicans *SC5314 after 24 hours of growth at 200rpm and: pH 4,5 in MM 1% glucose with a acid*

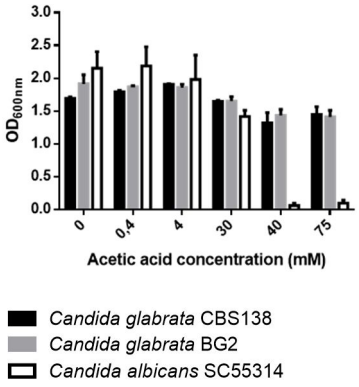

Figure 1 - Optical density at 600$_{nm}$ of *C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314 after 24 hours of growth at 200rpm and: pH 4,5 in MM 1% glucose with a acid

At low pH lactic and acetic acids do not synergistically inhibit growth of *Candida* spp.

Since acetic and lactic acids exist together in the vaginal tract, the occurrence of an eventual synergistic effect between these two acids in inhibiting growth of *Candida* spp was hypothesized. For these assays only pH 4 was tested. The results obtained (Figure 2) did not confirmed the proposed hypothesis since the presence of lactic acid did not augmented the strong toxic effect exerted by acetic acid. In fact, in the case of *C. albicans* SC5314, the presence of lactic acid slightly decreased tolerance of these cells to acetic acid which could be attributed to an eventual stimulation of lactate metabolism that could result in enhanced energy production used to counter-act the deleterious effects of acetic acid. The reduction oxygen availability yielded similar results (results not shown).

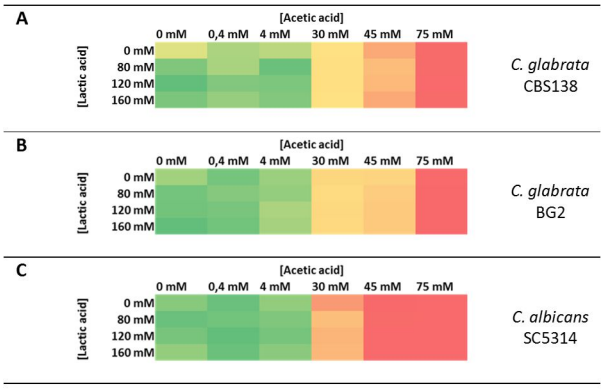

Figure 2- Heatmaps obtained thought the assays to assess the potential synergistic effect of lactic and acetic acids in inhibition of *Candida* growth in MM 1% glucose *at pH 4 after 24 hours at 20 rpm for C. glabrata* CBS138 (A), *C. glabrata* BG2 (B) and *C. albicans* SC5314 (C)

The presence of acetic and lactic acids at low pH modulates tolerance to azoles in *C. albicans and C. glabrata*

Since vaginal candidiasis is typically treated using topical azoles such as clotrimazole, miconazole, tioconazole and fluconazole, we have wondered whether the presence of acetic and lactic acids at a low pH could influence the activity of these antifungals. For that, the cells were cultivated in MM growth medium (having 1% glucose and adjusted at pH 4) supplemented with inhibitory concentrations of the different azoles and/or with acetic or lactic acids.

The results obtained showed that acetic acid exerted a synergistic effect with clotrimazole in inhibiting growth of *C. glabrata* CBS138 and *C. albicans* SC5314. This was particularly visible when *C. glabrata* CBS138 cells were cultivated in the presence of 40 mM acetic acid and 10 mg/L clotrimazole and during cultivation of *C. albicans* SC5314 in the presence of 40 mM acetic acid and 1 mg/L clotrimazole (Figure 3). Notably, this synergic effect between acetic acid and clotrimazole was not observed for the *C. glabrata* BG2 vaginal strain suggesting that it could be dependent on the genetic background of the strain. Lactic acid only showed a synergistic effect with clotrimazole for *C. albicans*, this effect being evident when cells were cultivated in the presence of 5mg/L of clotrimazole and 80 mM or in the presence of 10 mg/L clotrimazole and 80 mM lactic acid and in *C. glabrata* CBS138, in the presence of 160 mg/L and 160 mM of lactic acid No synergic effect between lactic acid and clotrimazole was observed for the two *C. glabrata* tested strains.

In the case of fluconazole, a slight synergistic effect was found with acetic acid for the *C. glabrata* CBS138 strain evident during cultivation in the presence of 60 mg/L of fluconazole and 40 mM of acetic acid. A similar effect was also observed for *C. albicans* SC5314, although in this case the effect is stronger and is already observed when cells are cultivated in the presence of 30 mg/L of fluconazole and 4 mM of acetic acid. Interestingly, all strains exhibited a reduced growth when cultivated in the presence of fluconazole and lactic acid suggesting that the presence of

this organic acid somehow seems to sensitize the cells against fluconazole.

In the case of miconazole a synergetic effect with acetic acid was observed for *C. glabrata* CBS138 in the presence of 30 mg/L of miconazole and 40 mM of acetic acid. For *C. albicans* a similar effect was observed when cells were cultivated in the presence of 0.4 mg/L of miconazole and 4 mM of acetic acid. Again this synergistic effect was not observed for the *C. glabrata* BG2 vaginal strain. When the medium was supplemented with lactic acid a clear synergistic effect was observed to occur with miconazole for *C. albicans* strain when cells were cultivated in the presence

.. Notably, no synergistic effect was observed for c. glabrata strains suggesting that the presence of these organic acids does not sensitize the cells against tioconazole

In the case of tioconazole , a synergistic effect was found with acetic acid just for *C. albicans* during cultivation in the presence of 0.6 mg/L of tioconazol and4 mM of acetic acid. When the cells are cultivated with tioconazole and lactic acid this effect was observed for C. albicans strain when just 0.1 mg/L of tioconazole and 80 mM of lactic acid were added to the medium of 0.2 mg/L of miconazole and 80 mM of lactic acid. It can be seen that when the medium was supplemented with 160 mM of lactic acid and 0.4 mg/L of miconazole a slight synergistic effect was also observed for *C. glabrata* CBS138 strain

Figure 3 - Graphic representation of the ODs obtained in the assays done to try to assess the potential synergistic effect of acetic acid with clotrimazole after 24h of growing in MM 1% glucose at 200 rpm for C. albicans SC5314.

Effect of acetic and lactic acids in adhesion of *C. glabrata* and *C. albicans* to abiotic surfaces

The modulation of adherence properties is an essential factor used by *Candida* spp to colonize different sites. In this part of the work the effect of the presence of lactic and acetic acids at low pH in the ability of *C. glabrata* and *C. albicans* to adhere to polystyrene was examined. For this cells were cultivated in MM 1% glucose medium (at pH 4) and the number of cells adhered to the surface of polystyrene was measured after 6H (results not shown) and 24h (Figure 4) using the crystal violet method. In the absence of the acids significant differences in the adhesion properties of the different strains were observed with the vaginal strain *C. glabrata* BG2 exhibiting a clear reduced number of adhered cells, especial when compared with the CBS138 strain. The *C. albicans* SC5314 strain was the one exhibiting a higher adhesive capacity, which is line with the reported (Tscherner et al. 2011)increased ability of this species to form biofilms, especially when compared with *C. glabrata* (Kucharíková 2016). Supplementation of the medium with acetic acid (0, 0.4, 30 and 45 mM) led to a slight reduction in the amount of biofilm formed by *C. glabrata* CBS138, this effect being more prominent for *C. albicans* (Figure 4 A). This is consistent with the above demonstrated high susceptibility of *C. albicans* SC5314 to acetic acid. Supplementation of the medium with lactic acid (0, 80, 120 and 180 mM) did not resulted in alterations in cell adhesion.

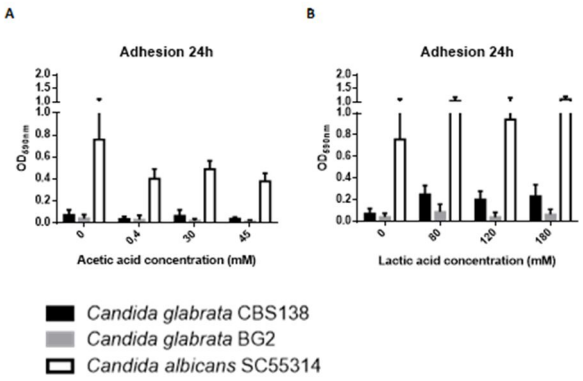

Figure 4- Results obtained by crystal violet method to assess the effect of acetic and lactic acids in adhesion of *C. glabrata* CBS138, *C.glabrata* BG2 and *C. albicans* SC5314 to abiotic surface (polystyrene). Results obtained at 590nm after growing in MM 1% glucose at pH4 with acetic acid after 24h with the supplementation with lactic acid after (A) and lactic acid (B) of growing.

Effect of the expression of *Ad1, Ad2*, Ad3 and Ad4 genes in *C. glabrata* tolerance to azoles

To examine the effect of the expression of *Ad1, Ad2*, Ad3 and Ad4 in *C. glabrata* tolerance to azoles the highly standardized EUCAST microdilution method was used [http://www.eucast.org]. To determine the concentration (designated MIC_{50}) of each drug that inhibited growth of each strain by more than 50%, in comparison with growth observed in drug-free medium. The assays were performed using RPMI growth medium adjusted at pH 4, differing from the protocol that is recommended by EUCAST in which the medium should be buffered at pH 7. However, the vaginal pH is acidic and therefore testing under such conditions is not very reasonable. The higher pH is also expected to drastically reduce the toxic potential of acetic acid. The results obtained showed that the individual deletion of the adhesin-encoding genes or of *CgHAA1* does not alter resistance of *C. glabrata*

to azoles (Table 1). Supplementation of the medium with acetic acid (30 mM) led to an increase in susceptibility of the wild-type KUE100 strain to fluconazole, tioconazole and miconazole (Table 1). This effect was augmented by the deletion of the *CgHAA1* gene (Table 1), presumably due to the effect that this gene has in conferring protection against acetic acid. The deletion of the adhesin-encoding genes also increased susceptibility of the cells to miconazole, however, this effect was much smaller than the one observed upon deletion of *CgHAA1*. It is not possible to establish if this effect results from the adhesin-encoding genes contributing to increase protection against the azoles or if it results from them contributing to increase protection against acetic acid.

Table 1 - MICS of azoles of *C. glabrata* KUE100, ΔHaa1, ΔAD1, ΔAD2, ΔAd3 and ΔAd4 obtained at pH4 and at pH4 supplemented with 30 mM of acetic acid

Azole		*C. glabrata* KUE100	*C. glabrata* Δhaa1	*C. glabrata* ΔAd1	*C. glabrata* ΔAd2	*C. glabrata* ΔAd3	*C. glabrata* ΔAd4
Clotrimazole	MIC pH 4	4 mg/L	4 mg/L	4 mg/L	4 mg/L	4 mg/L	4 mg/L
	MIC pH 4 + 30 mM acetic acid	4 mg/L	0.0625 mg/L	4 mg/L	4 mg/L	4 mg/L	4 mg/L
Fluconazole	MIC pH 4	64 mg/L	64 mg/L	64 mg/L	64 mg/L	64 mg/L	32 mg/L
	MIC pH 4 + 30 mM acetic acid	32 mg/L	0.125 mg/L	32 mg/L	32 mg/L	32 mg/L	32 mg/L
Miconazole	MIC pH4	1 mg/L	1 mg/L	1 mg/L	1 mg/L	1 mg/L	1 mg/L
	MIC pH 4 + 30 mM acetic acid	0.25 mg/L	0.0625 mg/L	0.125 mg/L	0.125 mg/L	0.125 mg/L	0.125 mg/L
Tioconazole	pH4	1 mg/L	1 mg/L	1 mg/L	1 mg/L	1 mg/L	1 mg/L
	MIC pH 4 + 30 mM acetic acid	0.25 mg/L	0.031 mg/L	0.25 mg/L	0.25 mg/L	0.25 mg/L	0.25 mg/L

To address this question the involvement of the adhesin-encoding genes in tolerance to acetic acid was also attempted, however, such analysis was not possible to complete this analysis during the course of this thesis

Effect of the expression of *Ad1, Ad2*, Ad3 and Ad4 genes in *C. glabrata* adherence to abiotic surfaces

The effect of the expression of the *Ad1, Ad2*, Ad3 and Ad4 genes in the ability of *C. glabrata* cells to adhere to the surface of polystyrene was examined in this part of the work. For this, cells of the different strains were cultivated in RPMI growth medium supplemented with increasing concentrations of acetic acid (4, 30 and 45 mM). The level of cells adhered to the surface of polystyrene was measured after 6 and 24h of cultivation using the crystal violet method. The increase in the concentration of acetic acid reduced adhesion of all strains, although this effect was more pronounced after 24h of growth when the biofilm is denser. Among the deletion strains tested only the mutant devoid of *CgHAA1* gene exhibited a reduced adhesion when cultivated in the presence of acetic acid (Figure 5), demonstrating that under these conditions the expression of the adhesins *Ad1, Ad2*, Ad3 and Ad4 is dispensable for biofilm formation

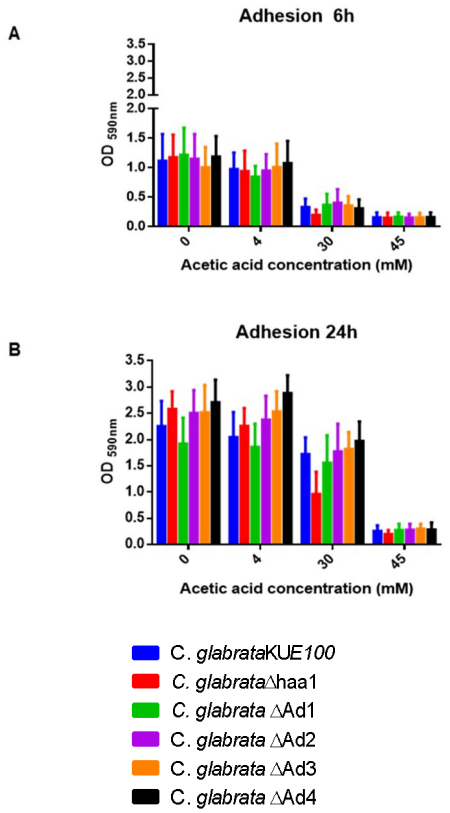

Figure 5 -Results obtained by crystal violet method to assess the effect of acetic acid in adhesion of *C. glabrata* strains to abiotic surface (polystyrene). Results obtained at 590nm after growing in RPMI 2% glucose with acetic acid after 6h (A) and 24h (B)

Effect of the expression of *Ad1*, *Ad2*, Ad3 and Ad4 genes in *C. glabrata* adherence to reconstituted vaginal human epithelium

The pathogenicity of *Candida* species is mediated by a number of virulence factors, including adherence and biofilm formation on host tissue (Silva et al. 2011). Adhesins are involved in specific adherence, so were performed assays with epithelial cells in order to try to evaluate the role of Ad1, Ad2, Ad3 and Ad4 adhesins in adhesion of *C. glabrata* to those biotic surfaces. The *Candida* and vaginal epithelial cells were co-cultured in RPMI 2% glucose at pH4 for 12 hours.

The results (Figure 6) show that even in the control situation in RPMI medium (without acetic acid) the expression all adhesins is important since the number of cells per tissue is reduced in the knock-out mutants, compared to the wild type strain. Supplementation of the medium with acetic acid reduced adhesion of all strains, with particular emphasis for strain devoid of *CgHAA1*. In the case of the other mutants the number of adhered cells was identical to the one observed in the acid-free medium suggesting that the lower adherence is not the result of the presence of the acid. On the overall, the results obtained show that the *Ad1, Ad2*, Ad3 and Ad4 genes important for *C. glabrata* adhesion and subsequent colonization of reconstituted vaginal human epithelial cells.

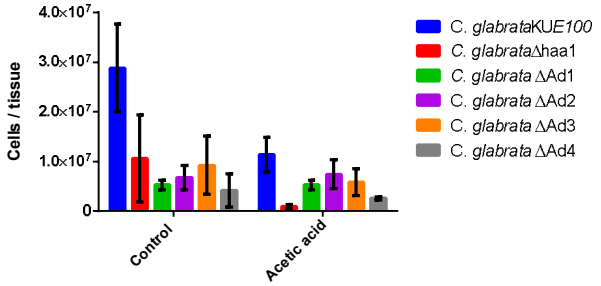

Figure 6 - Effect of acetic acid in cell adhesion of *C. glabrata* KUE100, Δhaa1, ΔAD1, ΔAD2, ΔAd3 and ΔAd4 after 12 hours in RPMI 2% glucose at pH4

Effect of the expression of genes Ad1, Ad2, Ad3 and Ad4 in *C. glabrata* virulence against the infection models *Caenorhabditis elegans* and *Galleria mellonella*

An eventual effect of *CgHAA1* and of the Ad1, Ad2, Ad3 and Ad4 genes in virulence of *C. glabrata* was assessed exploring *C. elegans* and *G. mellonela* as infection models. In the case of *C. elegans*, besides inoculation with the different *C. glabrata* strains, the worms were also inoculated with *E. coli* which was used as a control. The experimental methodology used was based on a previous study undertaken with *C. albicans* (Pukkila-worley et al. 2009; Pukkila-worley et al. 2011), as detailed in materials and methods. For this the *C. glabrata* cells were cultivated for 24h in solid BHI medium after which the plates were partially scrapped and the remaining cells used as the sole source of food for *C. elegans*. After 24h and under the conditions that were used about 50% of the worms died as a result of *C. glabrata* colonization (Figure 7). The wild-type KUE100 strain was the more virulent strain exhibiting the highest mortality rate, while the strain devoid of Ad3 gene was the less virulent one. Strains devoid of AD1 and of AWP3 were also found to be less virulent than the wild-type strain, although the effect was less pronounced. This trend in the mortality of the worms was maintained for longer periods of incubation. Despite the registered effect on the adhesin-encoding genes, the mortality induced by ΔCgHaa1 cells was identical to the one of the parental strain (Figure 7). The ΔAd4 was the *C. glabrata* mutant that registered the higher percentage of survival.

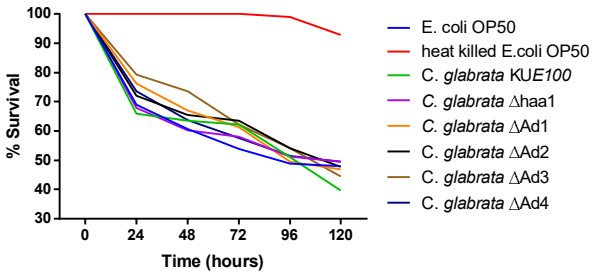

Figure 7 - Survival curves of *Caenorhabditis elegans* infected with *C. glabrata* KUE100 and *C. glabrata* Δhaa1 ΔAD1, ΔAD2, ΔAd3 and ΔAd4 (based on two replicates)

The effect of *CgHAA1* and of the Ad1, Ad2, Ad3 and Ad4 genes was also studied in *G. mellonella*, the results obtained being shown in Figure 8. The results obtained show that the deletion of *CgHAA1* significantly reduces virulence of *C. glabrata* against *G. mellonella*, being registered a decrease of 70% in the mortality rate. Strains devoid of Ad1 and Ad4 were also found to much less virulent than the wild-type strain KUE100, 66.7% and 66.7% of induced mortality, respectively.

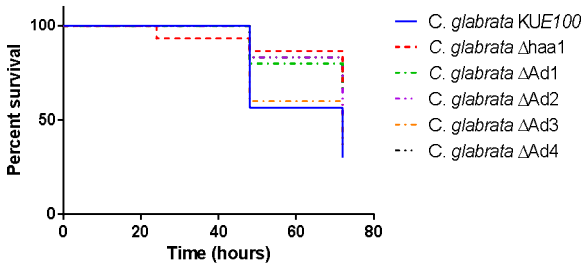

Figure 8- Kaplan–Meier survival curves of *Galleria mellonella* infected with *C. glabrata* KUE100, ΔCgHaa1, ΔAd1, ΔAd2, ΔAd3 and ΔAd4.

Discussion

The interest in the study of the effect on the physiology of *C. albicans* or *C. glabrata* caused by the presence of organic acids at a low pH has been boosted by the emerging descriptions of the essential role played by commensal bacteria, namely of Lactobacilii, in restraining the growth of vaginal pathogens. However, the studies undertaken regarding the effect of acetic and lactic acids in *C. albicans* and *C. glabrata* until so far had used pHs and/or acids concentrations which are different from those that are observed in the vaginal tract. In that sense, the herein described study differs by having applied a set of concentrations and pHs which are similar to those described to be present in the vaginal tract. We started to assess the inhibitory effect of the acetic and lactic acids in the growth of *C. albicans* SC5314, *C. glabrata* CBS138 and *C. glabrata* BG2 with a pH range from 4.5 to 3 and concentrations of acetic acid ranging from 0.4 to 80 mM and lactic acid ranging from 80 to 160 mM. The results obtained showed that all strains tested were not susceptible to the concentrations of lactic acid tested since no inhibitory effect was registered in all conditions studied. In fact growth with lactic acid was found to be even favorable for growth of the *Candida* strains. In the case of *C. albicans* this can be attributed to its ability to consume lactic acid even in the presence of glucose, a phenotypic trait that has been linked with the metabolic diversity and with the pathogenicity of this species (Childers et al. 2016; Ene et al. 2013). In the case of *C. glabrata* it has been described its ability to assimilate lactate when this is the sole carbon source but it has not been studied if this species is able to co-consume lactic acid when glucose was present in the growth medium, this being something that is suggested based on the results obtained in this study (Ueno et al. 2011). It is important to stress that recent results obtained in our research group show that *C. glabrata* is able to co-consume acetic acid in the presence of glucose (Cunha 2015) and thus it is very likely that the same could occur with lactic acid. Different from what was observed with lactic acid, exposure of the strains to acetic acid led to a significant inhibitory effect in growth, especially for the *C. albicans* SC5314 strain which was found to be highly susceptible. The higher tolerance of the two *C. glabrata* strains is in line with the generalized increased resilience of this species environmental stress, particularly when compared *with C. albicans* (Brunke & Hube 2013). It is not known whether this increased susceptibility that was herein observed is also observed in *C. albicans* vaginal strains or if it is somehow a trait specific of the SC5314 strain, which was isolated from the bloodstream. This is something that has to be further studied. On the overall the results obtained suggest that at least in vitro lactic acid has a far less pronounced significant effect in controlling growth of *Candida* spp than acetic acid. Necessarily the conditions utilized in our study did not simulate all those present in the vaginal tract and it might be possible that there is a condition that could somehow potentiate the toxic effect of lactic acid.

According to our results, the presence of acetic acid, the availability of glucose or of oxygen do not affect the overall increased resilience of the strains to lactic acid. In this context, it is possible that the levels of acetic acid maintained in the vaginal tract could contribute in a more significant manner for the control of the overgrowth of *Candida*. Part of this higher toxic effect of acetic acid could be linked to the normal vaginal pH that favors the acetic dissociation in higher concentrations since its pka is higher than the lactic acid pka. Another observation of relevance was the fact that the presence of lactic or acetic acids modulated tolerance of *C. albicans* and *C. glabrata* to azoles used to treat superficial candidiasis such as miconazole, tioconazole and fluconazole. This renders clear that the success in treatment of these vaginal infections could be largely dependent on the concentration of these organic acids that might be present in the vaginal tract with the presence of higher concentrations favoring the efficacy of the antifungal drugs. It is interesting that previously it has also been reported a synergistic effect of acetic acid with fluconazole and with caspofungin in inhibiting growth of *C. albicans* and *C. glabrata* (Ullah et al. 2013; Moosa et al. 2004). This discovery can have an especial interest in the development of novel vaginal candidiasis treatments. We have also tested whether the presence of these organic acids modulated adherence of the *Candida* cells to polystyrene and consequently their ability to form biofilms. Under the conditions used, only acetic acid was found to reduce adherence of *C. glabrata* and more significantly of *C. albicans* to form biofilms on the surface of PS. This observation is consistent with the above discussed higher susceptibility of this latter species to this organic acid. However, these results have to be further studied since the composition of the growth medium was found to greatly influence the capacity of the *Candida* cells to form biofilms on the surface of PS (Santos 2015). In this work this was also evidenced since formation of biofilm in the RPMI growth medium was much higher than the one observed with MM and the effect of acetic acid in inhibiting formation of biofilms was also more evident when the RPMI medium was used (compare figures Figure 4 and Figure 5). The presence of organic acids is likely to change the cell surface and consequently modulate adherence properties. Indeed, previous results have shown that exposure of *C. glabrata* cells to acetic acid or to benzoic acid leads to increased expression of several adhesin-encoding genes as well as genes involved in cell wall function (Bernardo et al. 2016; Mundy & Cormack 2009, Cunha 2014).

In the second part of this work it was performed the functional analysis of the *C. glabrata* uncharacterized adhesins genes *Ad1*, *Ad2*, Ad3 and Ad4 that were found to be up-regulated by acetic acid (Bernardo et al. 2016) , in the dependence of the CgHaa1 transcription factor. Specifically, we have examined the expression of the Ad1, Ad2, Ad3 and Ad4 genes in the ability of *C. glabrata* cells to adhere to the surface of polystyrene or to vaginal epithelial cells. According to the results obtained Ad1, Ad2, Ad3 and Ad4 adhesins are

dispensable for biofilm formation on the surface of PS in the presence of acetic acid. Nevertheless, the expression of these adhesins improved adhesion of *C. glabrata* to vaginal epithelial cells, this clearly demonstrating the necessity of having the "correct surfaces" to better perform the functional analysis of adhesins. This observation advances the current functional analysis of *C. glabrata* adhesins since up to now, only EPA1 and EPA6 adhesins had been implicated in adherence to vaginal cells (Mundy & Cormack 2009) . Although the deletion of the adhesins did not reduced the ability of *C. glabrata* to form biofilms in the surface of PS in the presence of acetic acid, a significant effect was observed upon CgHAA1 deletion. It is possible that under the conditions used CgHaa1 could regulate other adhesins or other genes that could be relevant for biofilm formation, this being a topic that will need further characterization in the future.

An eventual role of CgHaa1 and of Ad1, Ad2, Ad3 and Ad4 in determining virulence of *C. glabrata* against the infection models *G. mellonella* and *C. elegans* was also explored. The mechanism of nematode mortality during *C. glabrata* infection is unknown, but we show that *C. glabrata* is pathogenic to the nematode *C. elegans*. CgHAA1, as well as the adhesin Ad4 seems to be important for this enhanced *C. glabrata* virulence since a strain devoid of this gene exhibited a considerably lower rate of killing. Against *G. mellonella* it was also observed a reduced virulence of the strain devoid of CgHAA1. Two of the adhesins regulated by CgHaa1 were also found to have a reduced virulence against *G. mellonella* (Ad1 and Ad4) and thus it can be hypothesized that the reduced virulence can come from a lower ability of the DCgHaa1 strain to adhere to the host tissue. It remains to be tested whether there are other CgHaa1-regulated genes that could play a role in this determination of *C. glabrata* virulence. The demonstration that the CgHaa1 transcription factor is required for virulence of *C. glabrata* in two infection models in a condition where acetic acid is absent is highly interesting because it suggests that the pathway could have other activating signals.

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