TÉCNICO LISBOA

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.

Keywords: *C. glabrata, C. albicans, CgHAA1*, acetic acid, lactic acid, virulence

Resumo

Para que a colonização do nicho vaginal seja feita com sucesso, *C. glabrata* tem que superar vários stresses ambientais, os quais incluem, dentro de outros, a presença de ácido lático e acético que são produzidos pelas bactérias comensais da microflora. O pH acídico (~4) do tracto vaginal potencia o efeito tóxico desses ácidos carboxílicos, que se pensa que contêm o progresso de infeções causadas por agentes patogénicos, incluindo por *Candida* spp. Neste trabalho foi examinado como é que a presença de concentrações dos ácidos acético e lático afetam o crescimento de *C. glabrata CBS138, C. glabrata BG2* e *C. albicans* em pHs semelhantes aos encontrados no trato vaginal. Os resultados obtidos mostram que o ácido lático não exerce efeito inibitório significativo nas duas espécies testadas. Distintamente, o ácido acético inibiu o crescimento das duas espécies, contudo de forma mais acentuada em *C. albicans SC5314*. A modulação da tolerância aos azóis usados no tratamento de infeções superficiais foram afetadas pela presença dos ácidos acético e lático em pH baixo e a presença do ácidos acético e lático também contribuíram para modular a tolerância das estirpes ao polistireno.

Um segundo aspeto que foi desenvolvido neste trabalho, foi a análise funcional da via CgHaa1, que recentemente mostrou ser necessária para a máxima tolerância de *C. glabrata* ao stress causado pelo ácido acético. Mais especificamente, foi demostrando que um conjunto de adesinas encontrada que se descobriu serem reguladas por este factor de transcrição (Ad1, Ad2, Ad3 and Ad4) são necessárias para adesão e colonização das células epiteliais vaginais na presença do ácido acético. Além disso, foi também demonstrado que *CgHAA1* é necessário para a virulência de *C. glabrata* nos modelos de infeção *C. elegans* e *G. mellonella,* indicando que a função biológica deste sistema regulatório não está apenas envolvida em resposta ao ácido acético.

Palavras-chave: *C. glabrata, C. albicans, CgHAA1*, ácido acético, ácido lático, virulência

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Abbreviations

ABC	ATP-Binding Cassette
ATP	Adenosine-triphosphate
BHI	Brain Heart Infusion
BLC	Bacteriocin-like Compounds
EPA	Epithelial Adhesin
HCl	Hydrochloric ACID
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
NCAC	Non-*Candida albicans* species of *Candida*
OD	Optical Density
ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline
RHVE	Reconstituted Human Vaginal Epithelium
RNA	Ribonucleic Acid
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute medium
RVVC	Recurrent Vulvovaginal Candidiasis
VVC	Vulvovaginal Candidiasis

1. Introduction

The study of the human mycobiome has revealed the presence of a significant number of fungal species that colonize different niches including the genito-urinary (Bauters et al. 2002; Beigi et al. 2004; Ackerman et al. 2016), gastrointestinal (Tang et al. 2015) and respiratory tracts (Krause et al. 2016), as well as the oral cavity (Dupuy et al. 2014), the scalp or the skin. Among those fungal species that are known to colonize the human as commensals are species of the *Candida* genus namely *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei* (Mintz & Martens 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). Factors that have been described to underlie this switch from commensal to pathogenic state include alterations in the host environment such as physical insults to the mucosa, disruption of the indigenous microbiota by the use of antimicrobials, expression of specific virulence factors and access to site by excessive number of pathogens (Reid et al. 2011). Although infections caused by *Candida* (and by Fungi in general) are emerging throughout the world, these are still overlooked, specially when compared with infections caused by bacteria which have a much higher visibility. Due to their levels of recurrence and invasiveness *Candida* infections are associated with high rates of morbidity and mortality, being one of the more economic burdens for healthcare systems (Rosa et al. 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). 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) . In acidic niches, such as the vaginal tract (pH~4) (Aldunate et al. 2013) , these organic acids will exist mainly in their undissociated form, which has a well described microbiocidal effect (Piper 2011) . In the particular case of the vaginal tract, the bacterial microbiota is essentially composed by lactic acid bacteria which is believed to prevent the overgrowth of vaginal pathogens, including of *Candida* spp, through the production of carboxylic acids (Hickey et al. 2013; Hanlon et al. 2013; Parolin et al. 2015). A reduction in the activity of vaginal lactobacilii (such as the use of antibiotics) is a known-risk factor for the development of superficial and even of invasive candidiasis (Reid et al. 2011; Sze et al. 2014), clearly reflecting the essential role of the bacterial microbiota in the maintenance of vaginal health. 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*. Besides this, it was also investigated the role played by CgHaa1 regulon, a regulatory system recently described in our laboratory as being essential for *C. glabrata* to acetic acid (Bernardo et al. 2016), in virulence of this yeast species against vaginal epithelial cells and the infection models *Galleria mellonella* and *Caenorhabditis elegans*, thus extending the functional analysis of this regulatory pathway.

1.1. The role of vaginal microbiota in the control of vulvovaginal candidiasis

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 some cases, these vaginal infections can also progress to systemic infections since the vaginal tract provides an important driveway of access to the bloodstream (Kabir et al. 2012). 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). Although typically only one single species is identified as a causative agent of VVC, the occurrence of infections caused by two or more species has also been reported in a minority of women (1-10%) (Gonçalves et al. 2015). Notably it has been demonstrated that the same strains are behind episodes of recurrent vaginal infections, thus indicating that the predominant scenario is strain maintenance (Achkar & Fries 2010). It is presumed that this recurrence results from the strains evolving efficient adaptive responses that prevent them from being eradicated from the vaginal niche. In Table 1, adapted from Gonçalves *et al* 2015, it is highlighted the more relevant epidemiologic studies published during the last years concerning the distribution of the most common *Candida* species identified in women with VVC. Most of these studies reported higher association of *C. albicans* with VVC than with the remaining NCAC species (Gonçalves et al. 2015).

Table 1 - Epidemiologic studies published from 2003 to 2014, concerning the distribution of *Candida* species in women with VVC (Gonçalves et al. 2015).

Country	No. of subjects	*C. albicans* (%)	*C. glabrata* (%)	*C. tropicalis* (%)	*C. parapsilosis* (%)	*C. krusei* (%)
USA	429	75.7	15.6	1.1	3.8	3.8
Jamaica	125	83.2	4.8	11.2	–	–
Brazil	40	70.0	20.0	7.5	–	–
Italy	909	77.1	14.6	2.3	0.6	4.0
Slovakia	227	87.7	6.2	0.9	0.9	2.2
Austria	3243	87.9	3.4	0.2	0.5	0.3
Greece	576	80.2	7.3	1.6	2.1	1.0
Turkey	240	44.2	29.6	–	–	5.8
India	111	35.1	50.4	10.8	0.9	2.7
China	1070	90.4	7.9	0.9	0.6	0.1
Iran	175	65.1	13.1	6.2	0.6	4.0
Tunisia	295	76.3	19.3	1.4	–	–
Nigeria	517	20.3	33.8	17.9	4.3	–
Australia	1221	89.0	7.3	0.7	1.0	1.0

The microorganisms that compose the vaginal microbiota fluctuate with women's age, hormonal and immune status, sexual activities, use of medications, mode of contraception, and exposure to a variety of vaginal products (Ilkit & Guzel 2011). 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). *Lactobacillus iners, Lactobacillus crispatus, L. gasseri* and *L. jensenii* are the most prevalent species in the vaginal microbiota and their presence is well correlated with a healthy microbiota (Doerflinger et al. 2014; Borges et al. 2013). The vaginal microbiota of patients with diagnosed RVVC has been shown to be depleted from Lactobacilii, which further reinforces the idea that bacteria play an important role in maintaining vaginal health (Huang et al. 2011). The molecular mechanisms by which Lactobacilii inhibit the over-growth of *Candida* and other vaginal pathogens are not fully elucidated, although it is though to result, at least in part, from the production of lactic acid and/or acetic acid as the result of homo- or hetero- lactic fermentations (Mcdonald et al. 1987). 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). Besides the acidification of the vaginal environment per se, the presence of these organic acids in their undissociated form is also expected to be toxic since it they may enter microbial cells by simple diffusion where they can exert a panoply of negative effects. In the case of *C. albicans* and *C. glabrata* the molecular mechanisms of toxicity attributed to lactic and acetic acids involve ATP depletion, iron deprivation, intracellular acidification, ribosomal RNA degradation, among others (Mira, Teixeira, et al. 2010; Mollapour et al. 2008; Piper et al. 2001; Trček et al. 2015).

Up to now only a few proteins have been found to underlie tolerance to acetic acid in *C. albicans* and *C. glabrata,* as described in the following section. Nevertheless, these studies were performed in vitro directly adding the acids to the growth medium and it is not known if these mechanisms are also active when the *Candida* spp are cultivated directly in the presence of the *Lactobacilii*. Besides the production of organic acids, other described effects by which *Lactobacilii* can interfere with growth of *Candida* species include competition for nutrients and for adhesion sites and also the production of bacteriocin-like compunds (BLC), biosurfactants and hydrogen peroxide (H_2O_2) (Morales & Hogan 2010). Figure 1 shows how *Lactobacillus* sp., which normally inhabits the female reproductive tract, defends the host against colonization of pathogens such as *C. albicans*. Evidence suggests that the bacterium reduces the adhesion of *C. albicans* to epithelial cells either by (a) outcompeting fungal cells for adhesion sites, such as cellular receptors to which Lactobacillus has higher affinity, or (b) by secreting biosurfactants such as surlactin that physically decrease fungal binding. Most Lactobacillus strains release (c) hydrogen peroxide (H2O2) and (d) lactic acid or other fatty acids that inhibit *C. albicans* proliferation and invasive hypha formation. Bacteriocin-like substances (e) produced by *Lactobacillus* suppress the fungal growth to directly decrease its load (Morales & Hogan 2010)

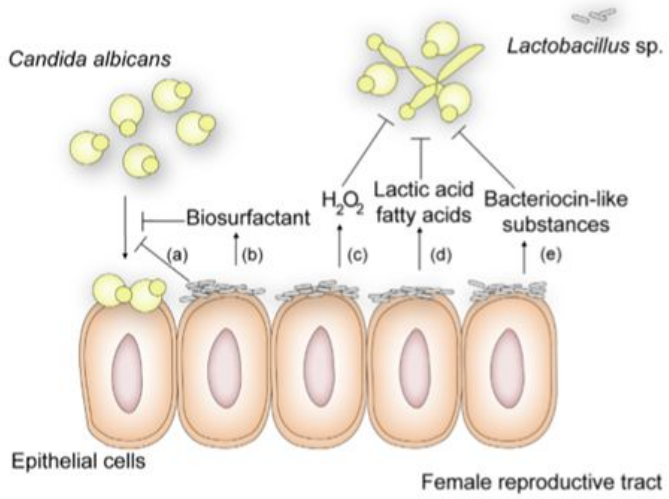


Figure 1- An overview of how *Lactobacillus* spp interacts with *C. albicans* in female reproductive tract (Morales & Hogan 2010).

1.2. Adaptive response and tolerance to the toxic effects exerted by acetic and lactic acids in Yeasts

Much of the knowledge gathered on response of yeasts to organic acids has been obtained in the experimental model yeast *Saccharomyces cerevisiae* (Mira, Teixeira, et al. 2010), however, in the more recent years some studies have also focused *C. glabrata* and *C. albicans* (Bernardo et al. 2016; Cottier et al. 2015; Eglè et al. 2014). When in aqueous solution weak acids partly dissociate leading to a dynamic equilibrium between the undissociated acid form and the anions/protons. At a pH below the acid pKa value, this equilibrium favors the undissociated acid form which is lipid-soluble and therefore able to penetrate the lipid bilayer of the plasma membrane by simple diffusion dissociating directly in the cytosol (which has a pH close to neutrality) (Stratford et al. 2013). In this sense, the antimicrobial potential of carboxylic acids is largely determined by their pKa and also hydrophobicity (Mira, et al. 2010).

Within the range of vaginal pH (3.5-4.5) both acetic acid (pKa=4.76) and lactic acids (pKa=3.86) will exist mainly in their undissociated form (Boskey et al. 1999; Ramos et al. 2016). For example at pH 4, approximately 80% of acetic acid and 42% of lactic acid are expected to be found in their undissociated form. The dissociation of the acid in the intracellular environment generates a proton and the corresponding acid anion (H^+, $XCOO^-$), which due to its negative charge cannot diffuse out of the cell (Mira, Becker, et al. 2010) (Mira et al. 2010). The accumulation of the negatively charged counter-ion causes several deleterious effects for the yeast cells including the increase in turgor pressure, oxidative stress, among others; these effects being largely dependent on the anion (Mira et al. 2010). In *S. cerevisiae* the internal accumulation of acetic acid has been shown to lead to growth inhibition, apoptosis, oxidative stress, inhibition of several metabolic enzymes (Piper et al. 2001; Abbott et al. 2009; Chen et al. 2016), while the accumulation of lactic acid was described to cause oxidative stress, programmed cell death, effects on cell membrane/wall composition and protein

aggregation (Abbott et al. 2009; Berterame et al. 2016). To reduce the internal accumulation of the acid anions *S. cerevisiae* cells rely on the activity of a set of inducible transporters mainly involved in multi-drug resistance (Figure 2). It is interesting to observe that depending on the acid anion, different transporters are involved: for example, Pdr12, belonging to the ABC superfamily, plays a crucial role in determining tolerance to propionic, benzoic and sorbic acids, but is largely dispensable for tolerance to acetic acid. Differently, Tpo3, Tpo2 and Aqr1, belonging to the MFS superfamily, are critical tolerance to acetic acid, but play a much less significant role in determining tolerance to benzoic or sorbic acids. In *C. glabrata* CgPdr12 (CAGL0M07293g) was found to also to play an important role in determining tolerance to sorbic acid and, more recently, it was shown that CgTpo3 contributes for maximal tolerance to acetic acid by helping to reduce the internal accumulation of this organic acid inside the cells (Bernardo et al. 2016; Mundy & Cormack 2009).

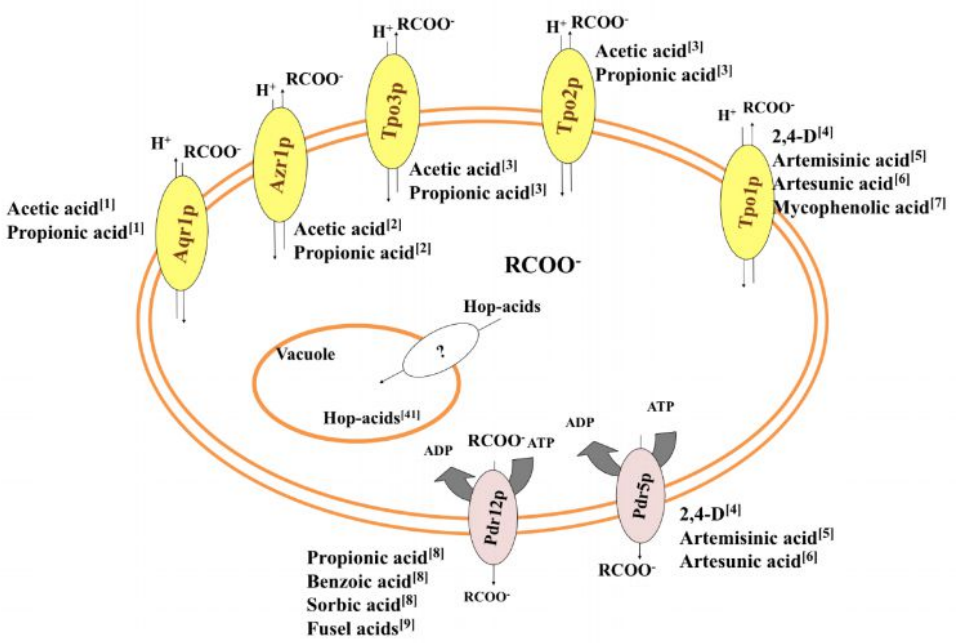


Figure 2 - Detoxification through multidrug resistance (MDR) transporters of the ATP binding cassette (ABC) (in yellow) and Major Facilitator Superfamily (MFS) (in pink) is required to reduce the internal concentration of the weak acid counterion (Mira et al. 2010).

The accumulation of protons upon dissociated of the acids in the cytosol was also found to play an important role underlying the toxic effect exerted by organic acids. Among other factors, intracellular acidification inhibits the activity of cytosolic metabolic enzymes and leads to the dissipation of the plasma membrane potential, which is an essential feature for secondary transport (Mira et al. 2010). Intracellular acidification was also reported to occur upon exposure of *C. glabrata* cells to inhibitory concentrations of acetic, benzoic and sorbic acids, albeit at a lower extension than the one observed to occur in S. cerevisiae (Ullah et al. 2013). To maintain the internal pH within physiological values, yeast cells rely on the stimulation of the activity of the plasma membrane H+-ATPase (PM-H+-ATPase), Pma1p, which couples ATP hydrolysis to

proton extrusion (Figure 3) (Mira, Teixeira, et al. 2010). In *C. glabrata* up-regulation of CgPma1 activity was also reported to occur under acetic acid and benzoic acid-challenge (Ullah et al. 2013; Bernardo et al. 2016).

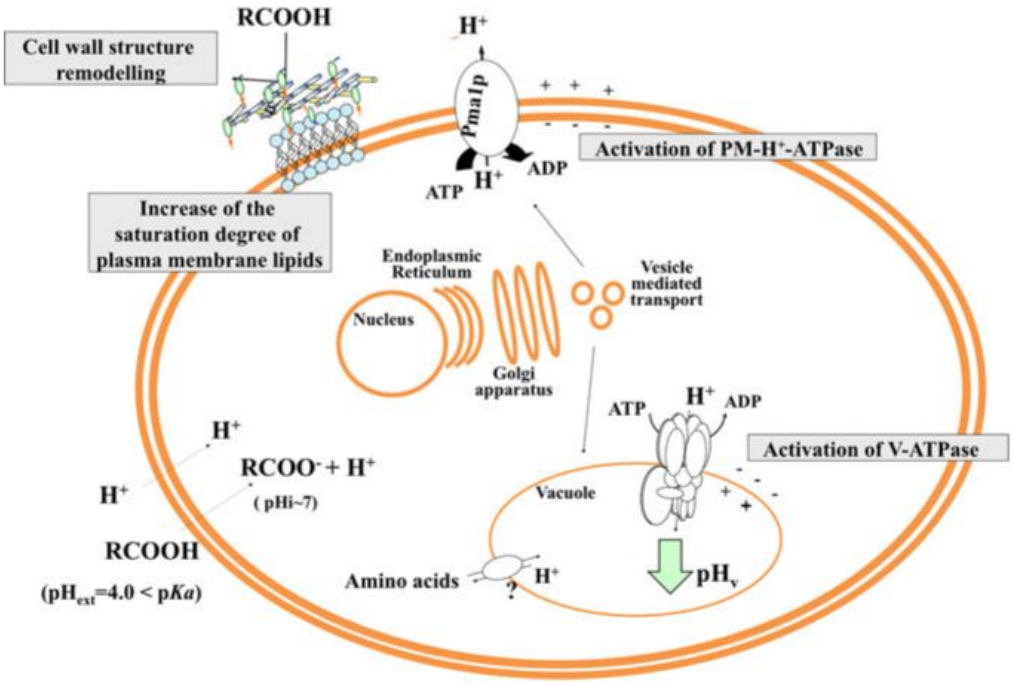


Figure 3 - Adaptive response in yeast cells: weak organic acids ability to cross plasma membrane, intracellular pH recovery and reconfiguration of cellular envelop (Mira et al. 2010).

1.3. Role of Haa1-regulon in determining Yeast's tolerance to acetic and lactic acids

In *S. cerevisiae* approximately 80% of the genes activated by acetic acid were found to be regulated, directly or indirectly, by the Haa1 transcription factor (Mira et al. 2011). Consistently, deletion of *ScHAA1* and of several genes activated by this transcription factor increased susceptibility of *S. cerevisiae* cells to acetic acid (Mira, Teixeira, et al. 2010). A particularly high susceptibility phenotype was obtained upon deletion of *ScTPO3* that, as said above, is involved in the reduction of internal accumulation of acetate/acetic acid; and of *ScHRK1*, encoding a protein with a poorly characterized function (Figure 4).

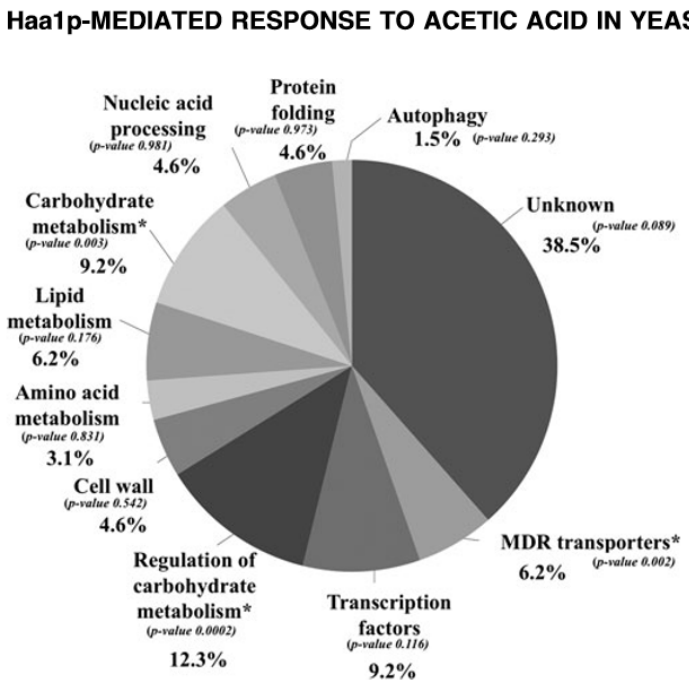


Figure 4 - Clustering, based on biological function, of genes activated in response to acetic acid stress in a Haa1 dependent way (Mira, Becker, et al. 2010).

Based on these evidences gathered in *S. cerevisiae* and also on the observation that *C. glabrata* genome encodes one homologue of *ScHaa1* gene (ORF CAGL0L09339g) in the iBB-BSRG laboratory was recently performed the functional analysis of this putative CgHaa1 (Bernardo et al. 2016). The results obtained clearly demonstrated the involvement of CgHaa1 in *C. glabrata* tolerance to acetic acid, being shown that this transcription factor regulates up 80% of the overall dataset of genes that are activated in acid-challenged cells (Bernardo et al. 2016). Among the genes found to be regulated by CgHaa1 are the multidrug resistance transporter CgTPO3; CgPMA1 (Figure 5), encoding the plasma membrane H^+-ATPase; CgHsp30 and CgPmp2, two predicted regulators of CgPma1 activity; and a group of 9 adhesins that have an uncharacterized function in *C. glabrata*. Consistently, it was demonstrated that the elimination of *CgHAA1* results in increased internal accumulation of acetic acid, in higher intracellular acidification and in reduced ability of *C. glabrata* to adhere and subsequently colonize reconstituted vaginal human epithelial cells(Bernardo et al. 2016). Altogether these results suggest that CgHaa1 could play a key role in increasing competitiveness of *C. glabrata* in the vaginal tract, especially in conditions of dysbiosis when the concentration of acetic acid increases in a very prominent manner (Bernardo et al. 2016).

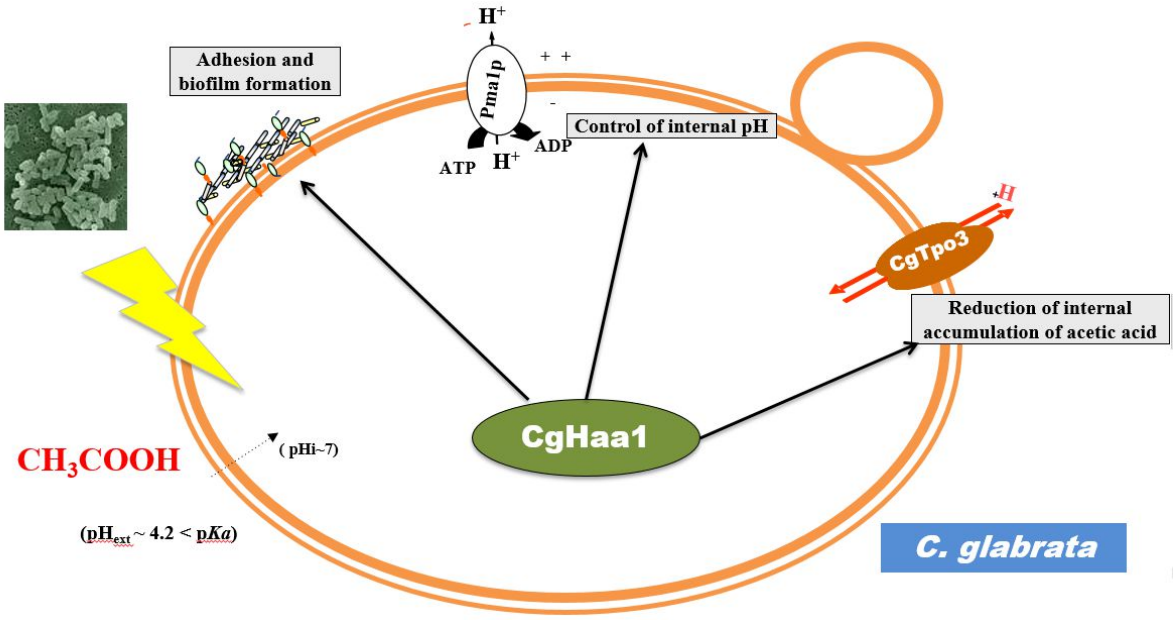


Figure 5 - Mechanistic model describing the main biological processes found to be under regulation of the CgHaa1-pathway under acetic acid stress in *C. glabrata*, according with the results described (Bernardo et al. 2016)

1.4. Introduction to the theme of the thesis

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. mellonella* and *C. elegans* was also scrutinized.

2. Materials and Methods

2.1. Strains, growth media and cultivation conditions.

The *C. glabrata* strains used are described in Table 2 and were kindly provided by Professor Hiroji Chibana from Chiba University in Japan. All the strains used were stocked at -80ºC in rich growth medium Yeast Peptone Dextrose (YPD) supplemented with 30% glycerol (V/V).

Table 2 - Strains of *Candida* species used.

Strain	Description
C. albicans SC5314	Reference strain; clinical isolate recovered from the blood of an infected patient
C. glabrata CBS138	Reference strain; clinical isolate recovered from human intestinal tract
C. glabrata BG2	Vaginal clinical isolate
C. glabrata KUE100	Wild-type strain derived from the CBS138 strain (Ueno et al. 2011)
C.glabrata Δhaa1	KUE100_ΔCAGL0L09339g

The *Candida* strains used were batch-cultured at 30°C, with orbital agitation (250 rpm) in minimal medium (MM), in rich yeast peptone dextrose (YPD), in RPMI and in BHI medium. MM contains, per liter, 1.70 g yeast nitrogen base (YNB) without amino acids and NH4+ (Difco Laboratories, Detroit, Mich.), 2.65 g (NH4)2SO4 (Merck Millipore) and glucose (20 or 10 g/L) (Merck Millipore, Darmstadt, Germany). YPD contains, per liter, 20 g glucose (Merck Millip3ore), 10 g yeast extract (HiMedia Laboratories, Mumbai, India) and 20g peptone (HiMedia Laboratories) and 30% glycerol (v/v) (Merck). RPMI (Roswell Park Memorial Institute Medium) contains, per liter, 10.8g RPMI-1640 synthetic medium (Sigma), 18g glucose (Merck Millipore) and 34,5g of MOPS (3-(N-morpholino) propanesulfonic acid, Sigma), in the case of RPMI 0,2% glucose no glucose was added. BHI contains 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).

2.2. Preparation of acetic and lactic acids 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.

2.3. Preparation of antifungals stock solutions

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.

2.4. Susceptibility assays undertaken in the presence of acetic or lactic acids.

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 stock solutions of the acids used to supplement the growth medium were 4x concentrated relative to the final concentration desired taking into account a necessary 1:4 dilution of the acid (50μL of the acid stock solution were applied in a total of 200 μL). The media and the organic acids concentrations were adjusted at 4 pHs: 4.5, 4, 3.5 and 3 using HCl as the acidulant. In Figure 6 it is schematically represented the experimental setting used. Each well contained 100 μL of the chosen growth medium (2x concentrated), 50 μL of the acid solution (4x concentrated; in control wells this volume was replaced by sterile water) and 50 μL of each cellular suspension. The cell suspensions (set an OD600 nm of 0.2) used as inocula were obtained by diluting a culture in mid-exponential phase (OD600nm of ~0.8). 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}.

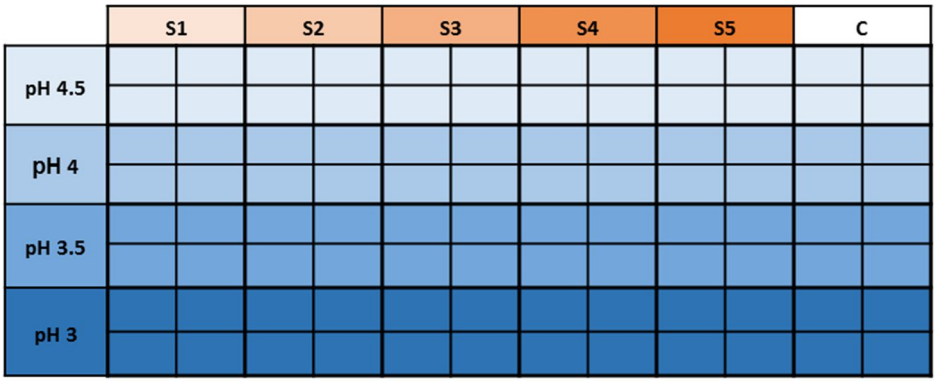


Figure 6 - Schematic representation of the strategy used to prepare the microplates in each susceptibility test performed to assess the potential inhibitory effect of acetic or lactic acids on growth of *C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314

2.5. Susceptibility assays undertaken in the presence of both acetic and lactic acids.

Susceptibility of *C. glabrata CBS138, C. glabrata BG2 and* C. albicans *SC5314* to the presence of both lactic acid and acetic acid was performed using an experimental setup similar to the one described above with the difference that in this case all solutions and media were adjusted to pH 4 only. The final concentrations of acetic acid tested in the microplates were those used before (0.4; 4; 30; 45; 75 mM) but in this case the stock solutions were 8x concentrated. The final concentrations of lactic acid in the microplates were 80, 120 and 160 mM and the corresponding stock solutions were also 8x concentrated. The microplates were prepared by adding 100 µL of each growth media (2x concentrated), 25 µL of each acid stock solution (8x concentrated) and 50µL of the cellular suspension also having a standardized OD_{600nm} of 0.2. The schematic representation of the microplate preparation is shown on Figure 7. As above, the plates were incubated at 30ºC (using an agitation of 200rpm or 20rpm) for 24h.

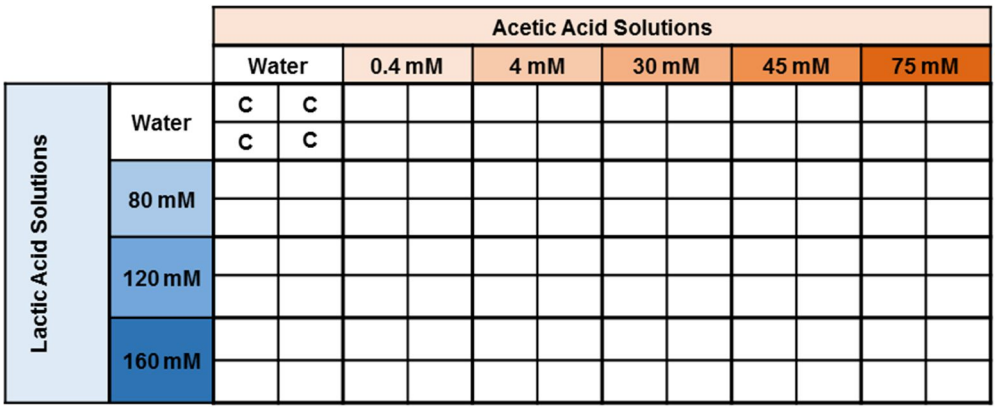


Figure 7 - Schematic representation of the strategy used to prepare the microplates in each susceptibility test performed to assess the potential inhibitory effect of acetic and lactic acids on growth of *C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314

2.6. Susceptibility assays undertaken in the presence of antifungals and acetic or lactic acids.

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 Thioconazol. In all cases the stock solution of the antifungal was adjusted at pH 4. The concentrations of acetic acid tested were 4, 40 and 75 mM for acetic acid, while for lactic acid it were tested 80, 120 and 160 mM. The stock solutions of the acids and of antifungals were 8x concentrated. To prepare the plates in each well it was added 100 µL of growth media (2x concentrated), 25 µL sterile water, 25 µL of the antifungal solution, 25 µL of the acid stock solution and 50 µL of cells' suspension. The schematic representation

of the microplate preparation is shown in Figure 8.. The plates were incubated at 30ºC (using an agitation of 200rpm) for 24h.

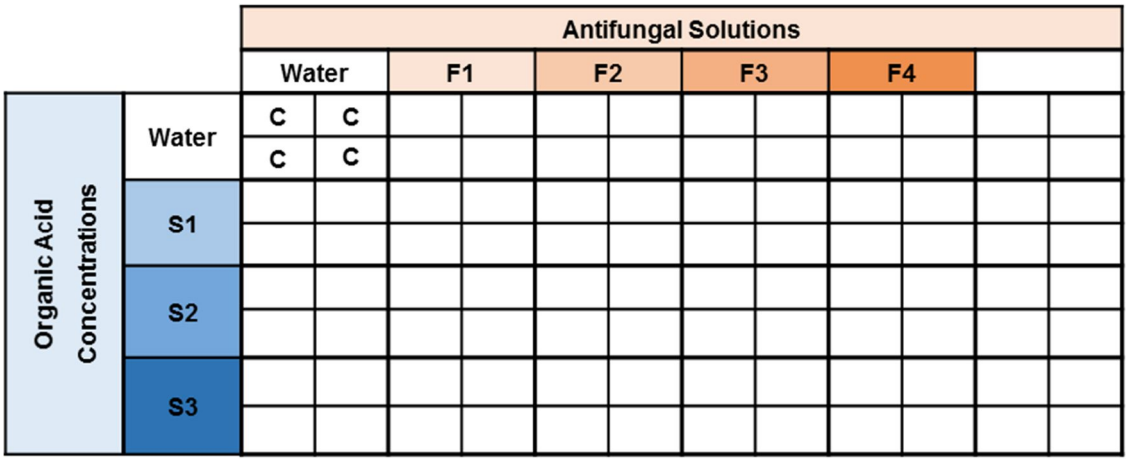


Figure 8 - Schematic representation of the microplate preparation for each growth media to assessment of the potential synergistic effect of acetic and lactic acids with antifungals on the growth of *C. glabrata* CBS138, *C. glabrata* BG2 *and C. albicans* SC5314.

2.7. Effect of acetic acid and lactic acid in adhesion of *C. albicans* and *C. glabrata* to polystyrene

The effect of the presence of acetic or lactic acids in the ability of C. albicans, *C. glabrata* BG2 and *C. glabrata* CBS138 to adhere to polystyrene was monitored after 6 h of incubation in mm 1% glucose growth medium not supplemented with the acids in 96-multiwell plates. For this cells of the different species were cultivated until mid-exponential phase (OD_{600nm} approximately 0.8), diluted to an initial OD600nm of 0.2 and finally inoculated in the 96-multiwell plate yielding an initial OD of 0.1. After 6 h and 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).

2.8. Effect of the expression of *CgHAA1* and of the CgHaa1-regulated genes Ad1, Ad2, Ad3 and Ad4 in susceptibility of *C. glabrata* to acetic acid and to azoles

The comparison of susceptibility to azoles was undertaken using the highly standardized microdilution method recommended by EUCAST (http://www.eucast.org) to determine the minimum inhibitory concentration. Briefly, *C. glabrata* KCHr606 cells and the derived deletion mutants ΔAWP12,

ΔAWP13, 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, as schematically represented in Figure 9. Notice that the assays were performed at normal pH and pH 4. Growth control wells (column 11) containing medium drug free were also inoculated with 100 µL of cellular suspension. A sterile control (column 12) contain 100 µL of drug-free medium and100 µL of sterile water was also performed. The microplates were incubated without agitation at 37ºC during 24h after which the absorvance at 590nm was measured.

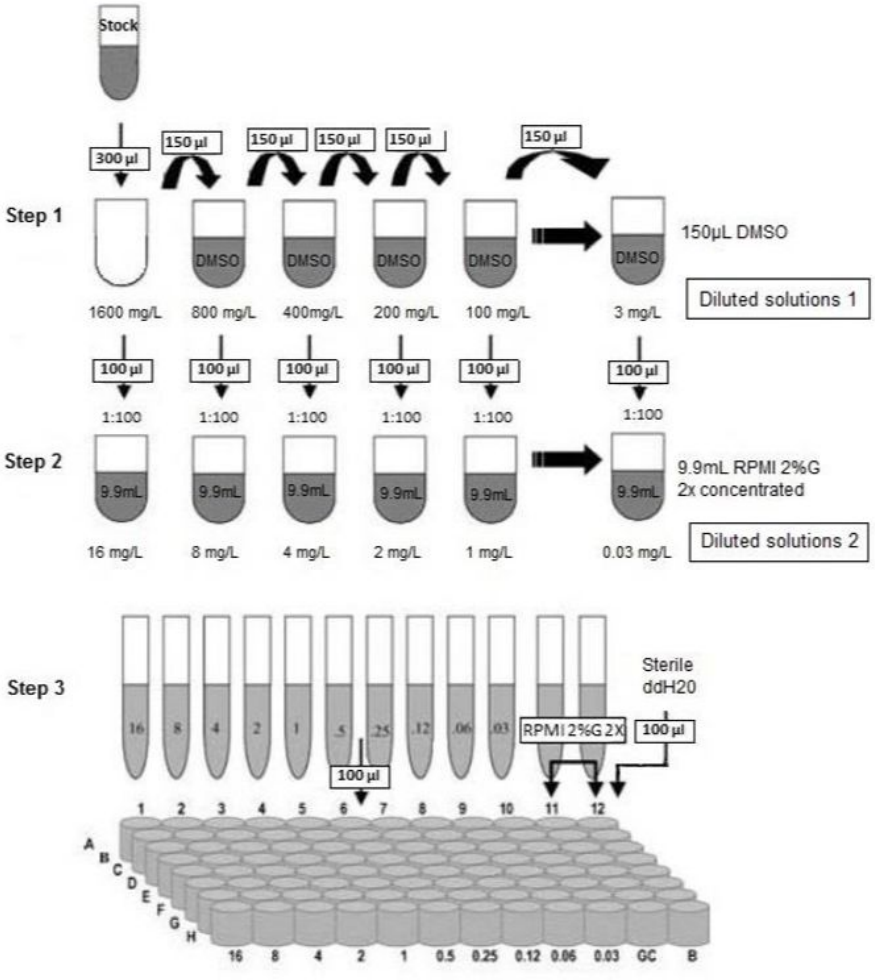


Figure 9 - Schematic representation of the procedure used to prepare the 96-multiwell plates used to determine MIC of voriconazole, anidulafungin and caspofungin for the different *C. glabrata* isolates. B: blank; GC: control. Image altered from EUCAST discussion.

The stock solutions of the antifungals used were prepared from the powder in DMSO after which subsequent dilutions were prepared. The range of concentrations tested is shown in Table 3. The MIC_{50} of the azoles where also determined using RPMI medium (adjusted at pH 4)

supplemented with 30 mM acetic acid. In this case 50 µL of 30 mM of acetic acid (2x concentrated) where added to 100 µL of RPMI 2% glucose at pH 4. The remaining steps were identical to the above described.

Table 3 - Concentration ranges for antifungal agents used to the determination of MICs.

Antifungal drug	**Range (mg/L)**
Fluconazole	0.125–64
Miconazole	0.06–32
Tioconazole	0.03–16
Clotrimazole	0.06–32

2.9. Effect of the expression of *CgHAA1* and of the CgHaa1-regulated genes *Ad1, Ad2*, Ad3 and Ad4 in adherence of *C. glabrata* to polystyrene and to reconstituted vaginal human epithelium

The adhesion of wild-type *C. glabrata* KUE100 cells or of the derived deletion mutants ΔAWP12, ΔAWP13, ΔAd3 and ΔAd4 to polystyrene was compared using the crystal violet method and following the same procedure described above.

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.

To study the effect of acetic acid in infection of vaginal epithelium by KUE100, ΔHaa1 ΔAWP12, ΔAWP13, ΔAd3 and ΔAd4 cells a commercially available reconstituted human vaginal epithelium (RHVE) (SkinEthic 335 Laboratories; Nice, France) was used as in vitro model of vaginal candidosis. The method used is described by (Alves et al. 2014). RHVE tissues were inoculated 24 h with 1 ml of standardized suspensions of the two *C. glabrata* strains in RPMI medium adjusted to pH 4 (about 2×106 cells/ml) either or not supplemented with 30 mM acetic acid. As a control, two RHVE tissue preparations incubated only with 1 mL RPMI or RPMI and acetic acid were prepared. All the infected tissues were incubated at 37°C in a 5% CO2 environment in saturated humidity for respective time. After incubation, the tissue was rinsed twice in 1 ml of PBS to remove non-adherent *Candida* cells, and the tissue was then bisected, with one half being used for fluorescence microscopy analysis and the other for molecular studies. For fluorescence microscopy analysis the tissue preparations were fixed in 2% (v/v) formalin and stored at 4°C until histological processing. Tissues were then dehydrated, cleared, and infiltrated with paraffin wax embedding material. The formalin-fixed, paraffin-embedded (FFPE) tissues were stored at room temperature. The tissues were cut (5 µm sections) and placed on Histobond+ coated microscope slides (Raymond A Lamb, East Sussex, UK), de-waxed, and processed through xylene, ethanol, and water before peptide nucleic acid probe hybridization. Peptide nucleic acid probe fluorescence in situ hybridisation (PNA FISH) was employed on tissue sections using the Light PNA FISHTM kit (AdvanDx Inc., Woburn MA, USA). This species-specific probe was used to study the colonization of the RHVE by *C. glabrata*. The Light PNA FISHTM kit had previously been developed and evaluated using multicolour labelled fluorescent PNA probe targeting specific 26S rRNA sequences of *C. glabrata* (Alves et al. 2014b). Tissue sections on microscope slides were overlaid with 1 drop of the respective PNA probe. After 90 min of incubation, in the dark in a humidified chamber at 55°C, unbound probe was removed by washing the slides using a previously warmed wash solution at 55°C for 30 min. The preparation was then mounted with a medium suitable for fluorescence microscopy (Vectashield, Vector laboratories, California, USA). Tissue sections (5 µm) hybridized with PNA probes were observed by fluorescence microscopy, using a BX51 Olympus fluorescence microscope with a DP71 digital camera coupled (Olympus Portugal SA, Porto, Portugal) to analyze the level of colonization in presence or absence of 30 mM of acid acetic of the surface of RHVE tissues. Quantification of *Candida* cells in the different tissue preparations was performed based on the quantification of genomic DNA. For this, the infected tissues were placed in a sterile 1.5

mL microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) with approximately 300 µL of 367 glass beads (0.5 mm diameter - Sigma, St. Louis, Mo.) and 600 µL of sorbitol buffer (GRiSP, Porto, Portugal). This final mix was homogenized three times for 60 s, using a Mini-Beadbeater-8 (Stratech Scientific, Soham, UK). After tissue disruption, the supernatant was carefully removed and placed in another sterile microcentrifuge tube. Then, DNA extraction was performed using the GRS Genomic DNA kit – Tissue (GRiSP), 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, Wilmington, DE, USA). *C. glabrata* genomic DNA was quantified using real-time PCR in a CF X96 Real-Time PCR System (Bio-Rad, Berkeley, USA). Each reaction mixture consisted of 10 µl of working concentration of SsoFast EvaGreen Supermix (Bio-Rad, Berkeley, USA), 0.2 µl of each primer (50 µM) designed previously (forward- ATTTGCATGCGCTTGCCCACGAATCC and reverse- GGTGGACGTTACCGCCGCAAGCAATGTT), and 4 µl of DNA, in a final reaction volume of 20 µl. Negative controls were performed using a reaction mixture with dH2O (Cleaver Scientific Ltd, UK) substituting for the template DNA. Template DNA for each positive control was obtained from FFPE tissues after the step of DNA extraction described above. 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 at 60°C was run to generate a melting curve for confirming the specificity of the amplification product. Previously, a calibration curves (Ct vs. Log cells) for each *C. glabrata* strain were constructed using the same PCR protocol as described above. For these, serial dilutions of the *Candida* cells were prepared and the DNA for PCR analysis extracted from the planktonic cell pellet using the DNA extraction kit (QIAamp® DNA FFPE Tissue, Qiagen, Crawley, UK) with some modifications (Bernardo et al. 2016).

2.10. Effect of the expression of *CgHAA1* and of the CgHaa1-regulated genes *Ad1, Ad2*, Ad3 and Ad4 in *C. glabrata* virulence against *C. elegans*

The effect of the expression of *CgHAA1* and of the CgHaa1-regulated genes *Ad1*, *Ad2*, Ad3 and Ad4 in virulence of *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, ΔAWP12, ΔAWP13, 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.

2.11. Effect of the expression of *CgHAA1* and of the CgHaa1-regulated genes *Ad1, Ad2,* Ad3 and Ad4 in *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 ΔAWP12, ΔAWP13, 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.

3. Results

3.1. *C. glabrata* and *C. albicans* are differently susceptible 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. The concentrations of glucose have been found to vary considerably on different infection sites where *Candida* cells grow being reported to be around 0.07–0.13% in the bloodstream and about 0.5% in the vaginal tract (Childers et al. 2016). Two strains of *C. glabrata* were used for this: the reference 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 (Figure 10A and B) 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. 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.

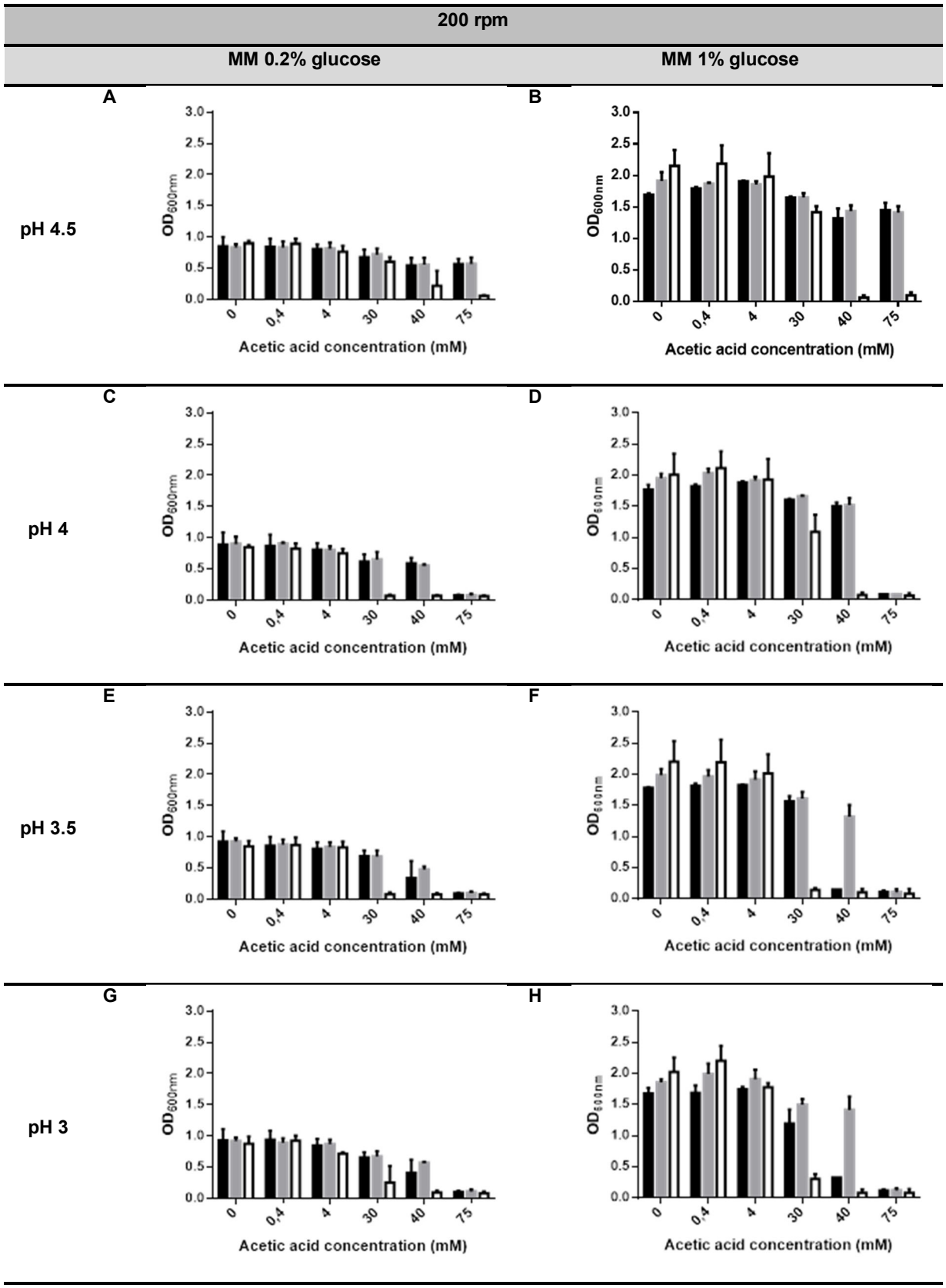


Figure 10 - Optical density at 600_{nm} of *C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314 after 24 hours of growth with acetic acid at 200rpm at: pH 4,5 in MM 0.2% glucose (A) and MM 1% glucose (B); pH 4 in MM 0.2% (C) and MM1 % (D); pH 3.5 in MM 0.2% (E) and MM1 % (F) and pH 3 in MM 0.2% (G) and MM1 % (H).

Exposure of the three *Candida* strains to lactic acid did not resulted in inhibition of growth, independently of the concentration of acid used or of the medium pH (Figure 11). In fact, in some cases (more prominent for *C. glabrata* CBS138 and *C. glabrata* BG2) 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).

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. The results (Figure 12 and Figure 13) obtained confirmed the higher susceptibility of *C. albicans* to acetic acid, with the two *C. glabrata* strains showing a higher resilience. In general there were no significant differences 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.
As observed 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 (Figure.13).

On the overall the results obtained show that *C. albicans* SC5314 is much more susceptible to acetic acid than *C. glabrata*, this effect being potentiated by the acidification of the medium presumably due to the increase in the concentration of the toxic undissociated form of the acid. Under the conditions used, lactic acid did not exerted any inhibitory effect in growth, its presence in fact being beneficial to improve growth. To exclude the possibility that these results could be related with the utilization of a minimal medium all the experiments were repeated with RPMI (results shown in Annex A Figure S1 and Figure S2). The results obtained were, in general, the same as those obtained with the MM medium.

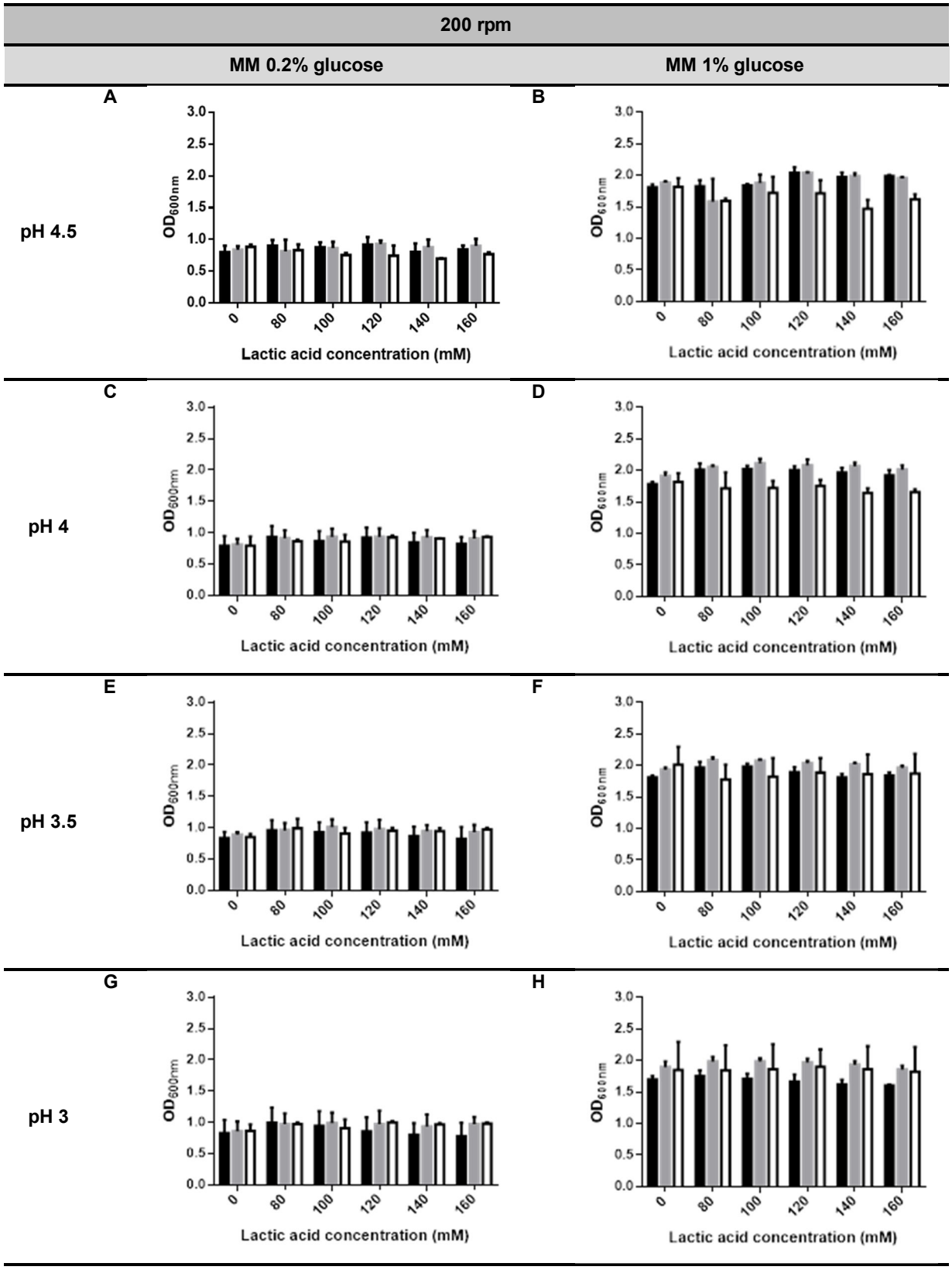


Figure 11 - Optical density at 600_{nm} of *C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314 after 24 hours of growth with lactic acid at 200rpm at: pH 4,5 in MM 0.2% glucose (A) and MM 1% glucose (B); pH 4 in MM 0.2% (C) and MM1 % (D); pH 3.5 in MM 0.2% (E) and MM1 % (F) and pH 3 in MM 0.2% (G) and MM1 % (H).

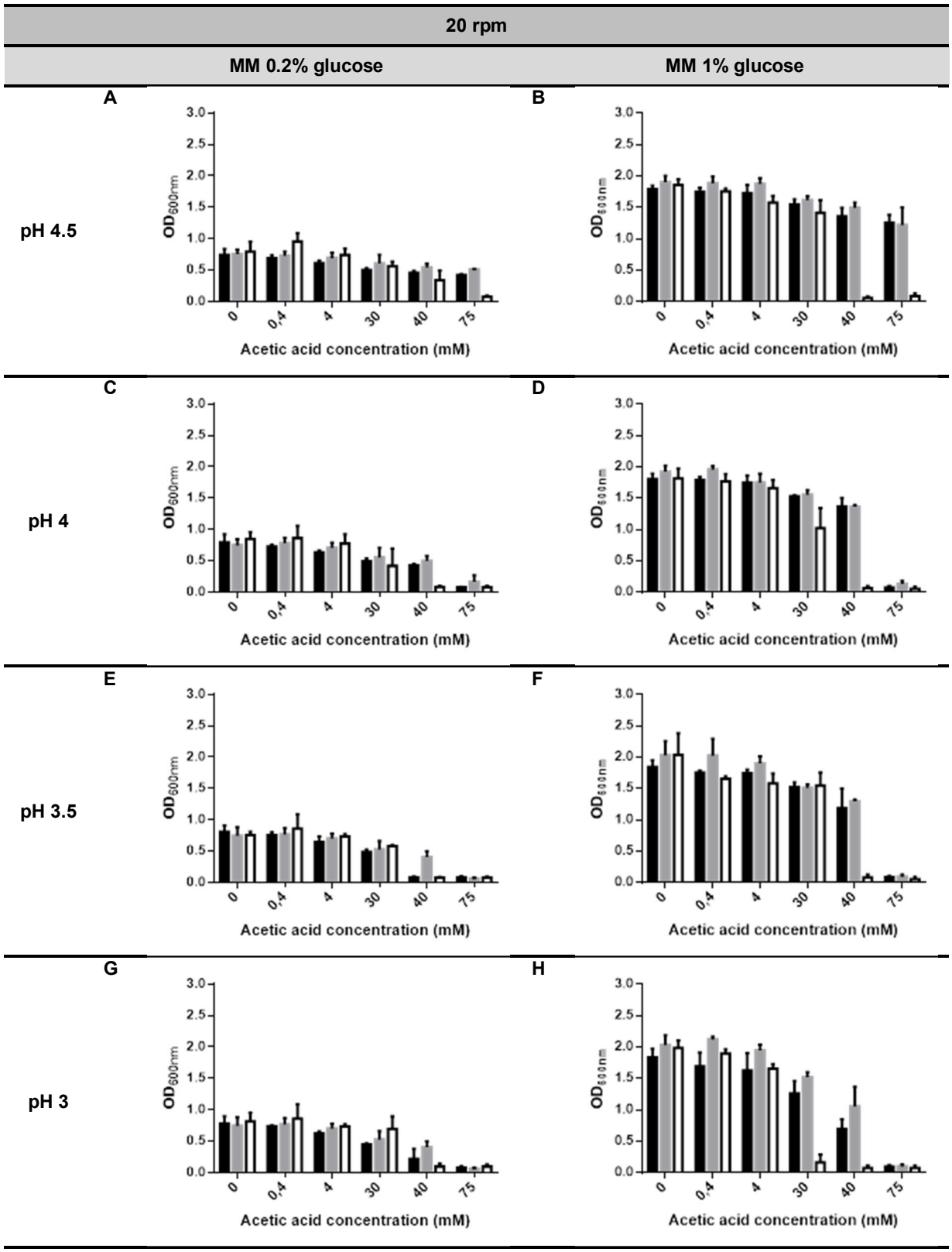


Figure 12 - Optical density at 600_{nm} of *C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314 after 24 hours of growth with acetic acid at 20rpm at: pH 4,5 in MM 0.2% glucose (A) and MM 1% glucose (B); pH 4 in MM 0.2% (C) and MM1 % (D); pH 3.5 in MM 0.2% (E) and MM1 % (F) and pH 3 in MM 0.2% (G) and MM1 % (H).

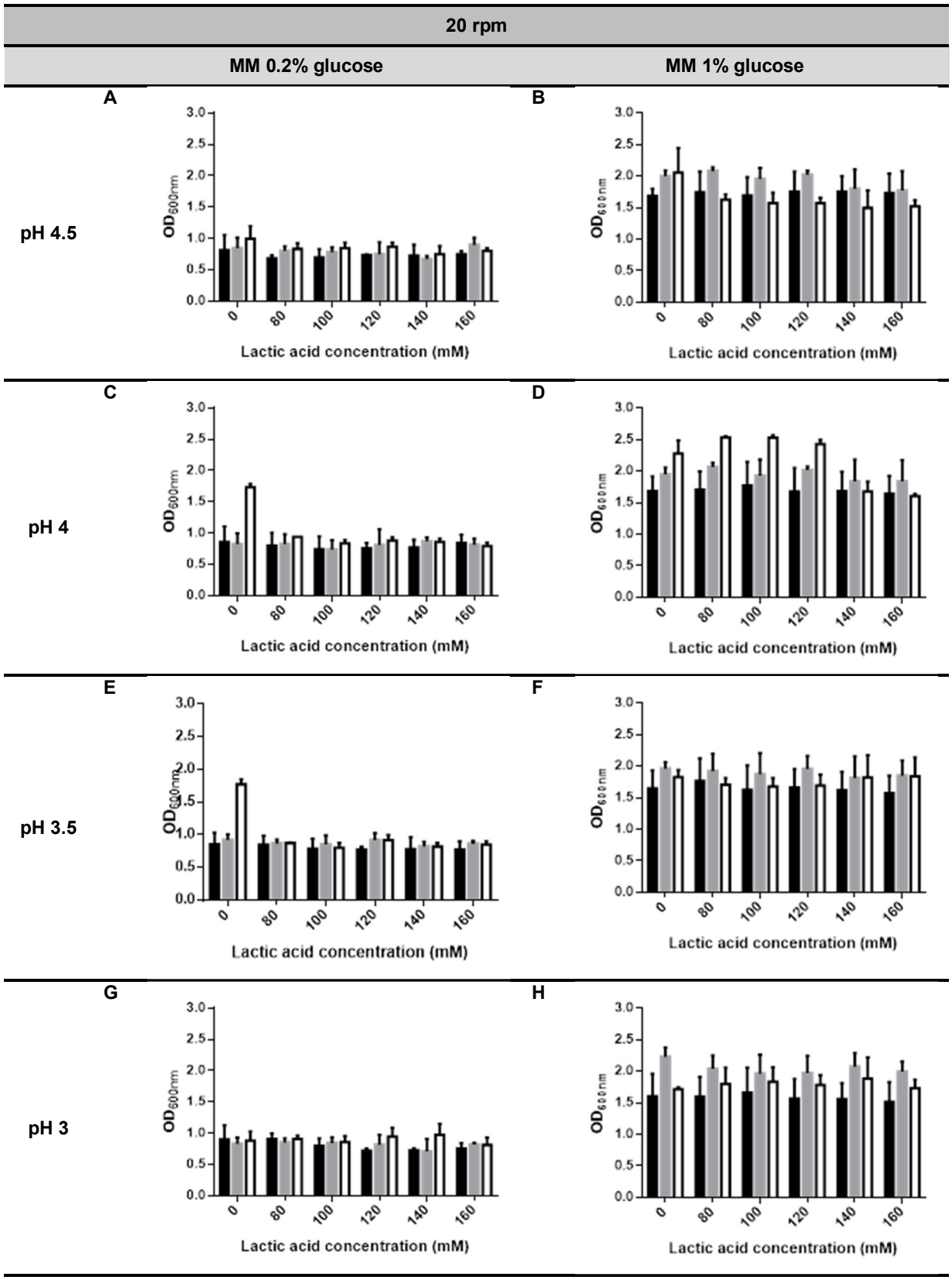


Figure 13 -- Optical density at 600_{nm} of *C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314 after 24 hours of growth with lactic acid at 20rpm at: pH 4,5 in MM 0.2% glucose (A) and MM 1% glucose (B); pH 4 in MM 0.2% (C) and MM1 % (D); pH 3.5 in MM 0.2% (E) and MM1 % (F) and pH 3 in MM 0.2% (G) and MM1 % (H).

3.2. 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. To test this, the strains were cultivated under the same experimental conditions described above with the difference that this time both acetic and lactic acids were simultaneously added to the growth medium. For these assays only pH 4 was tested. The results obtained (Figure 14) 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.

To have a more clear view on the data obtained and to render clear the idea that there is no synergist effect on growth inhibition between acetic and lactic acids, in Figure 15 it is shown the results obtained after 24h of growth in the different conditions, that is, in the presence of acetic acid only, in the presence of lactic acid only or in the presence of the two acids. The assays were also performed in MM 0,2% glucose and in RPMI 2% glucose, and are shown in Annex B (Figure S3 and S4 respectively)

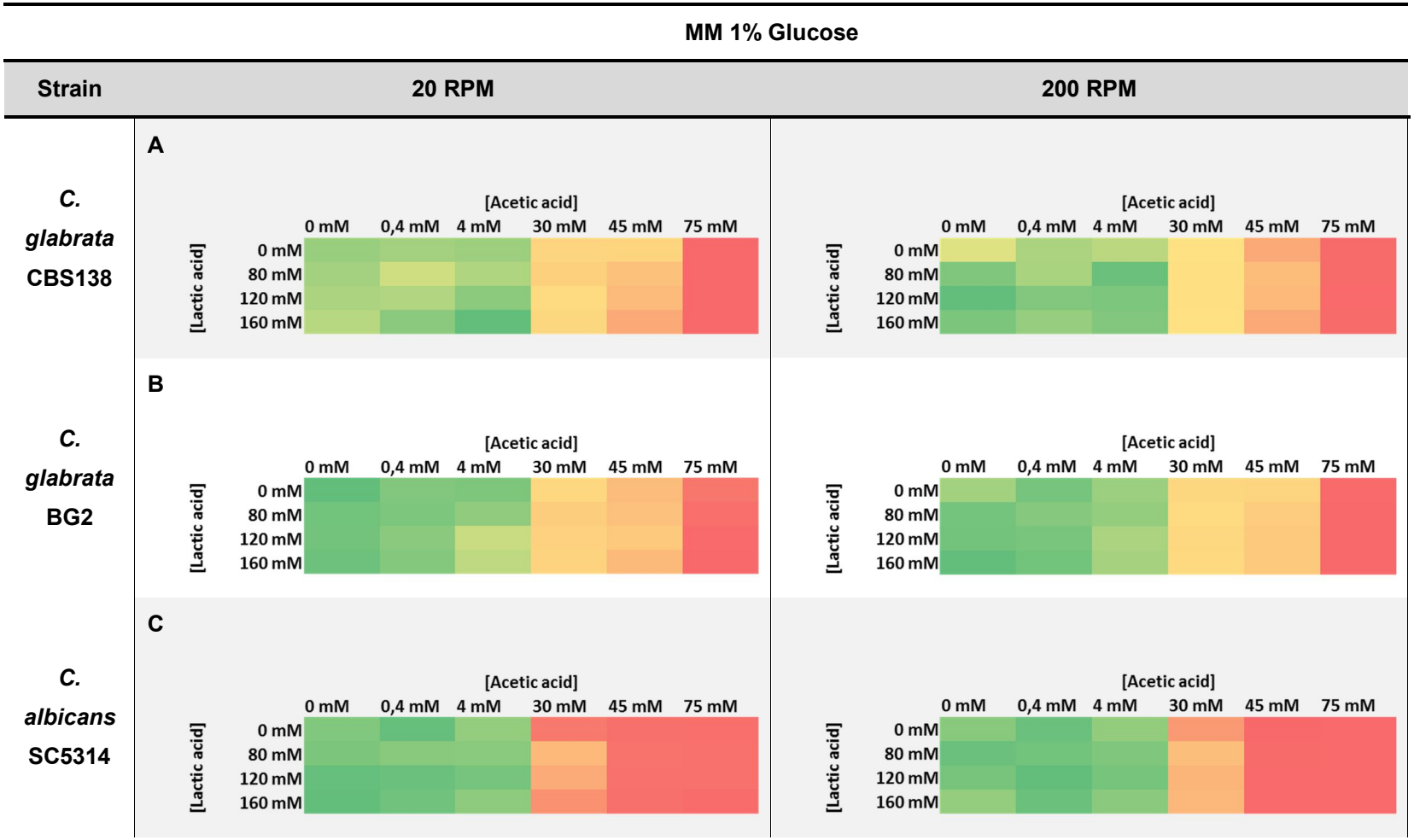
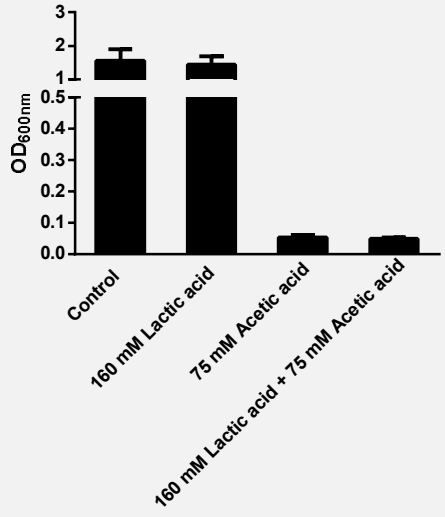
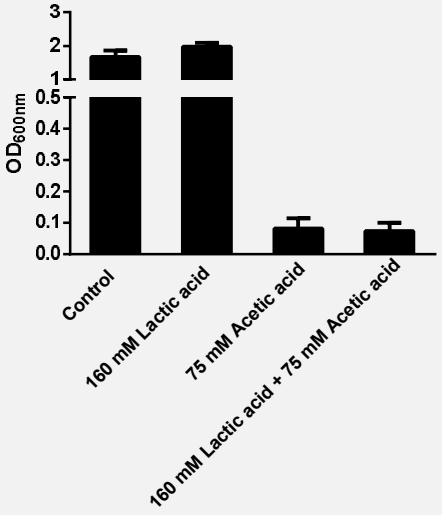
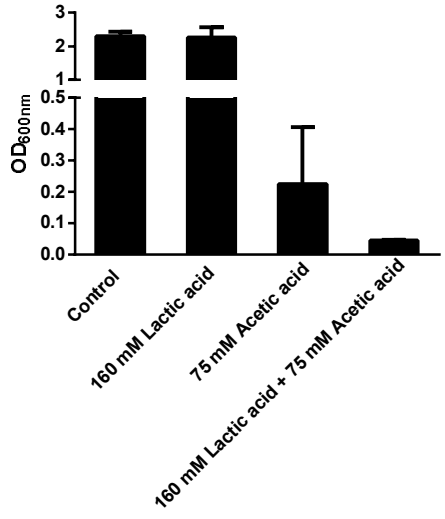
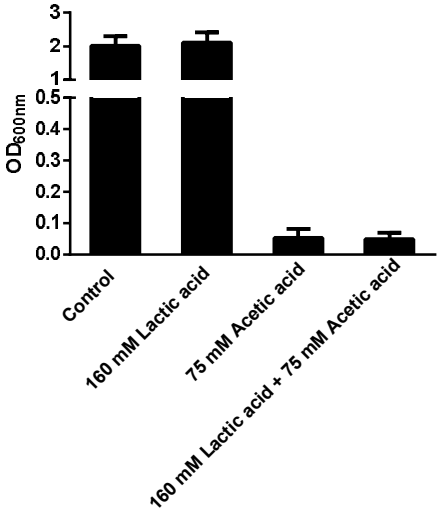


Figure 14 - Heatmaps obtained thought the assays to assess the potential synergistic effect of lactic and acetic acids in inhibition of *Candida* growth in MM 1% glucose at *pH 4 after 24 hours at 20 rpm for C. glabrata* CBS138 (A), *C. glabrata* BG2 (C) and *C. albicans* SC5314 (E) and *at 200 rpm for C. glabrata* CBS138 (B), *C. glabrata* BG2 (D) and *C. albicans* SC5314 (F). More dark green means more growth and more red more inhibition. Each heat map is formatted according it biggest value of DO.

	MM 1% Glucose	
Strain	**20 RPM**	**200 RPM**
C. glabrata **CBS138**	**A** 	**B** 
C. glabrata **BG2**	**C** 	**D** 

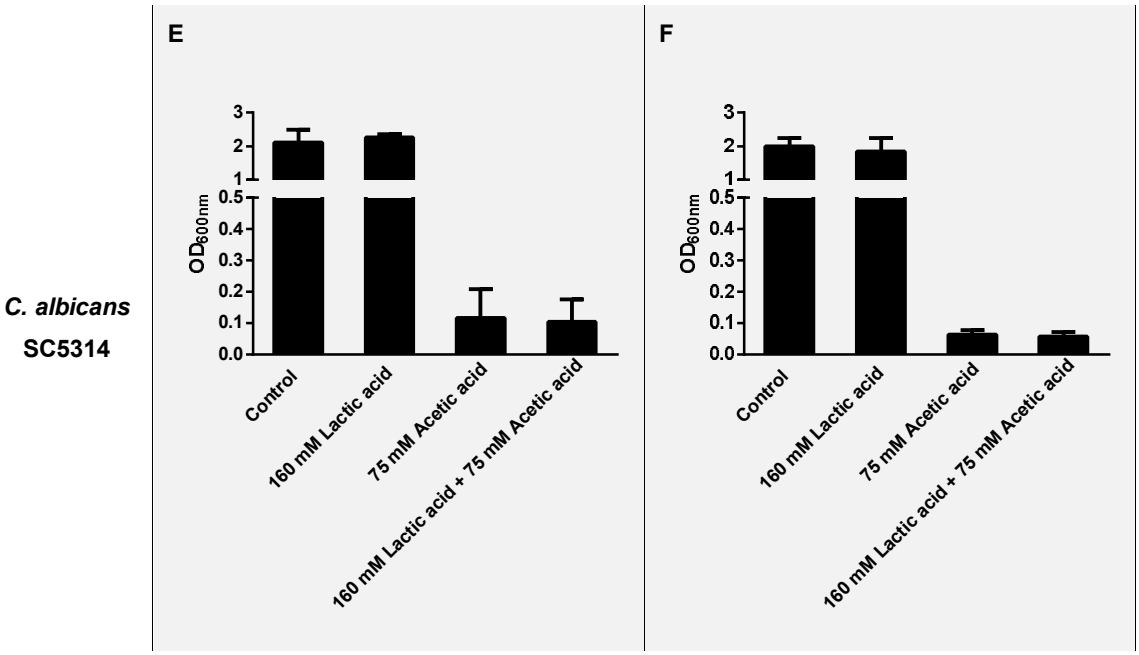


Figure 15 - Graphic representation of the results done to try to assess the potential synergistic effect of lactic and acetic acids after 24h of growing in MM 1% glucose for *C. glabrata* CBS138 at 20 rpm(A) and 200rpm (B), for *C. glabrata* BG2 at 20 rpm (C) and 200 rpm (D) and for *C. albicans* SC5314 at 20 rpm (E) and 200rpm(F).

3.3. 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 (Figure 16) or lactic (Figure 17) 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 (Figure 16A and C). 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 18A and C). 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 (Figure 16B and Figure 18B). 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 (Figure 22C) and 80 mM lactic acid and in *C. glabrata* CBS138, in the presence of 160

mg/L and 160 mM of lactic acid (Figure 22A) No synergic effect between lactic acid and clotrimazole was observed for the two *C. glabrata* tested strains (Figure 17B and Figure 22B).

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 (Figure19A). A similar effect was also observed for *C. albicans* SC5314 (Figure 16E), 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 (Figure19C). Interestingly, all strains exhibited a reduced growth when cultivated in the presence of fluconazole and lactic acid (Figure 23) 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 (Figure 20A). 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 (Figure 20C). 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 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 24A).

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 and 4 mM of acetic acid (Figure21C). 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 (Figure 25C). 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.

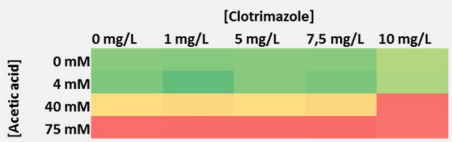
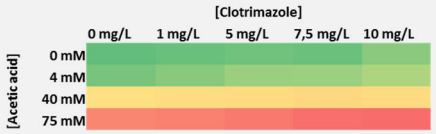
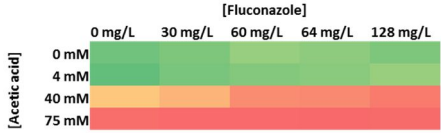
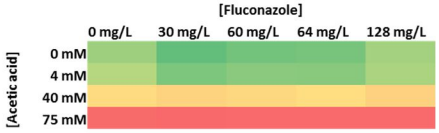
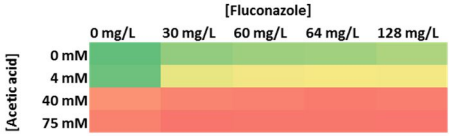
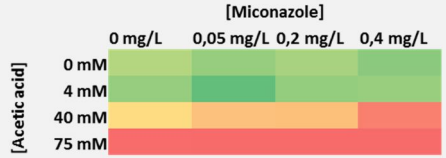
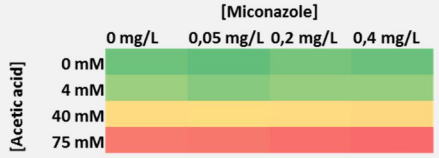
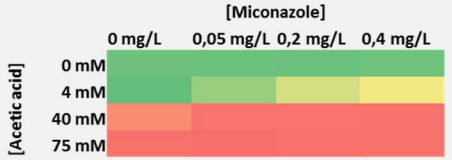
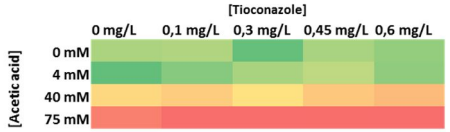
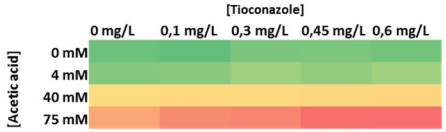
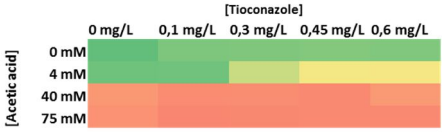
Azole	*C. glabrata* CBS138	*C. glabrata* BG2	*C. albicans* SC5314
Clotrimazole	**A** 	**B** 	**C** 
Fluconazole	**D** 	**E** 	**F** 
Miconazole	**G** 	**H** 	**I** 
Tioconazole	**J** 	**K** 	**L** 

Figure 16 - Heatmaps obtained through ODs obtained at 600nm, after 24h of growing in MM 1% at 30 ºC at 200rpm.To asses the potential synergistic effect between acetic acid and clotrimazole in *C. glabrata* CBS138 (**A**), *C.glabrata* BG2 (**B**) and *C. albicans* SC5314 (**C**).Effect of acetic acid and fluconazole in *C. glabrata* CBS138 (**D**), *C.glabrata* BG2 (**E**) and *C. albicans* SC5314 (**F**). Effect of acetic acid and miconazole in *C. glabrata* CBS138 (**G**), *C.glabrata* BG2 (**H**) and *C. albicans* SC5314 (**I**). Effect of acetic acid and tioconazole in in *C. glabrata* CBS138 (**J**), *C.glabrata* BG2 (**K**) and *C. albicans* SC5314 (**L**).

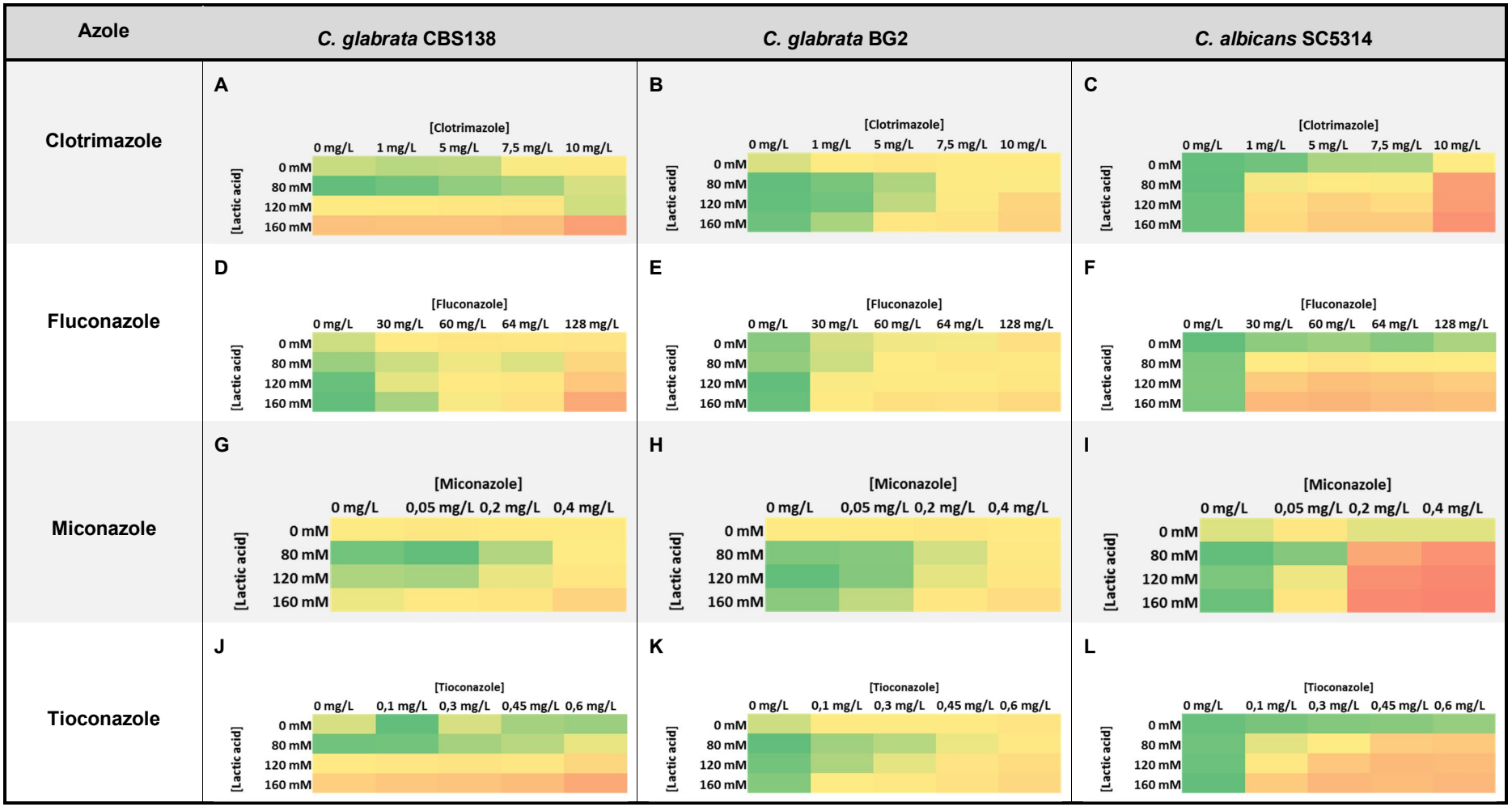


Figure 17 - Heatmaps obtained through ODs obtained at 600nmm, after 24h of growing in MM 1% at 30 ºC at 200rpm. To assess the potential synergistic effect between lactic acid and clotrimazole in *C. glabrata* CBS138 (**A**), *C.glabrata* BG2 (**B**) and *C. albicans* SC5314 (**C**).Effect of acetic acid and fluconazole in *C. glabrata* CBS138 (**D**), *C.glabrata* BG2 (**E**) and *C. albicans* SC5314 (**F**). Effect of acetic acid and miconazole in *C. glabrata* CBS138 (**G**), *C.glabrata* BG2 (**H**) and *C. albicans* SC5314 (**I**). Effect of acetic acid and tioconazole in in *C. glabrata* CBS138 (**J**), *C.glabrata* BG2 (**K**) and *C. albicans* SC5314 (**L**).

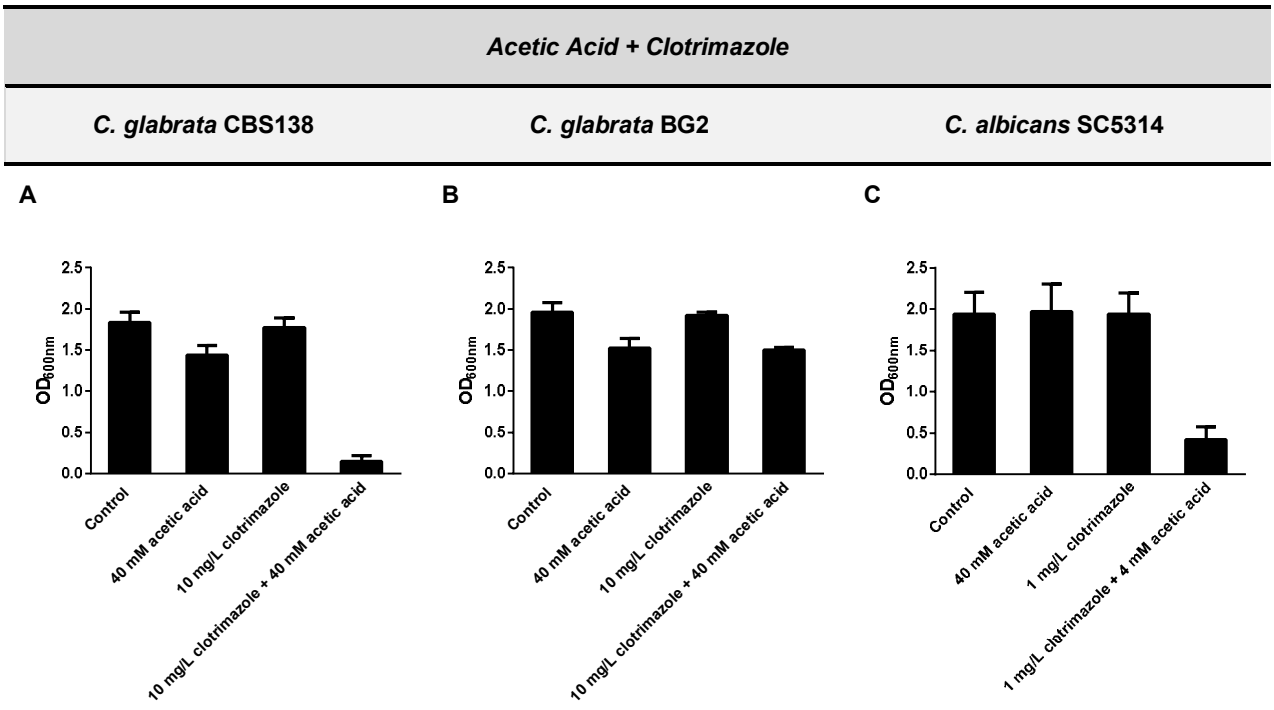


Figure 18 - Graphic representation of the ODs obtained in the assays done to try to assess the potential synergistic effect of acetic acid with clotrimazole after 24h of growing in MM 1% glucose at 200 rpm for *C. blabrata* CBS138 (A), *C. glabrata* BG2 (B) and C. albicans SC5314 (C).

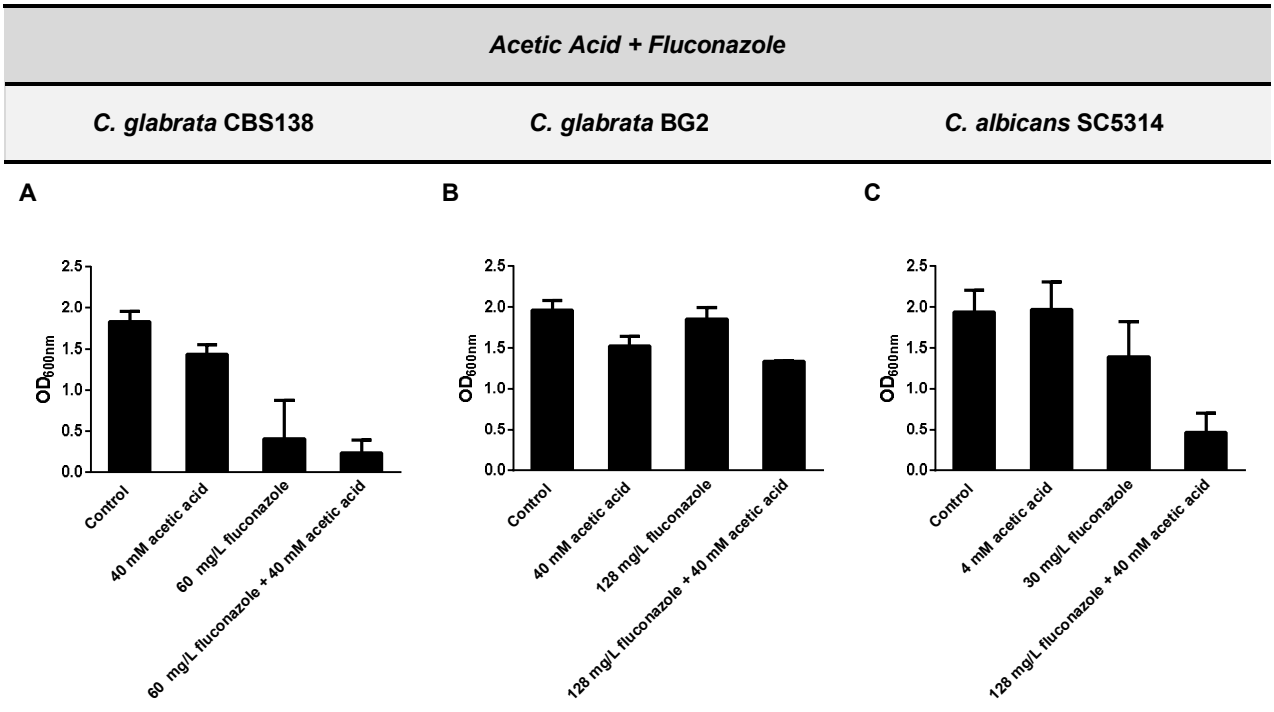


Figure 19 - Graphic representation of the ODs obtained in the assays done to try to assess the potential synergistic effect of acetic acid with fluconazole after 24h of growing in MM 1% glucose at 200 rpm for *C. glabrata* CBS138 (A), *C. glabrata* BG2 (B) and *C. albicans* SC5314 (C).

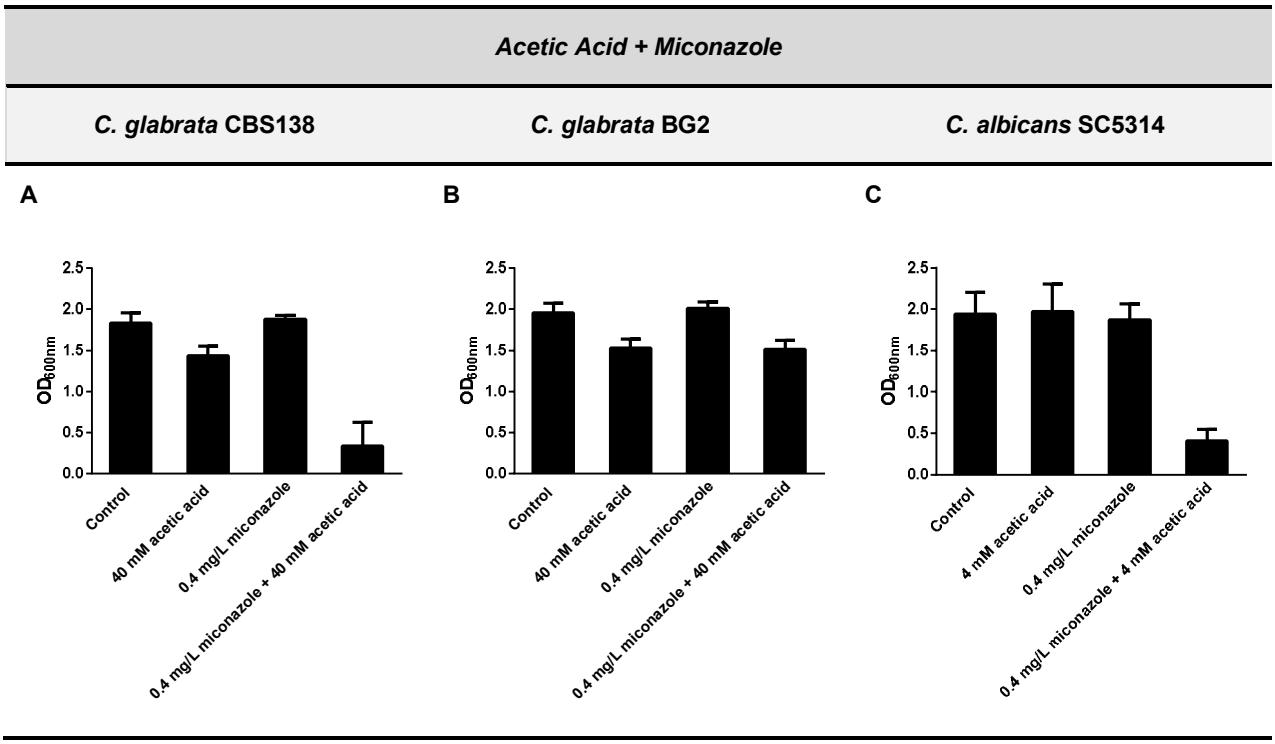


Figure 20 - Graphic representation of the ODs obtained in the assays done to try to assess the potential synergistic effect of acetic acid with miconazole after 24h of growing in MM 1% glucose at 200 rpm for *C. blabrata* CBS138 (A), *C. glabrata* BG2 (B) and C. albicans SC5314 (C).

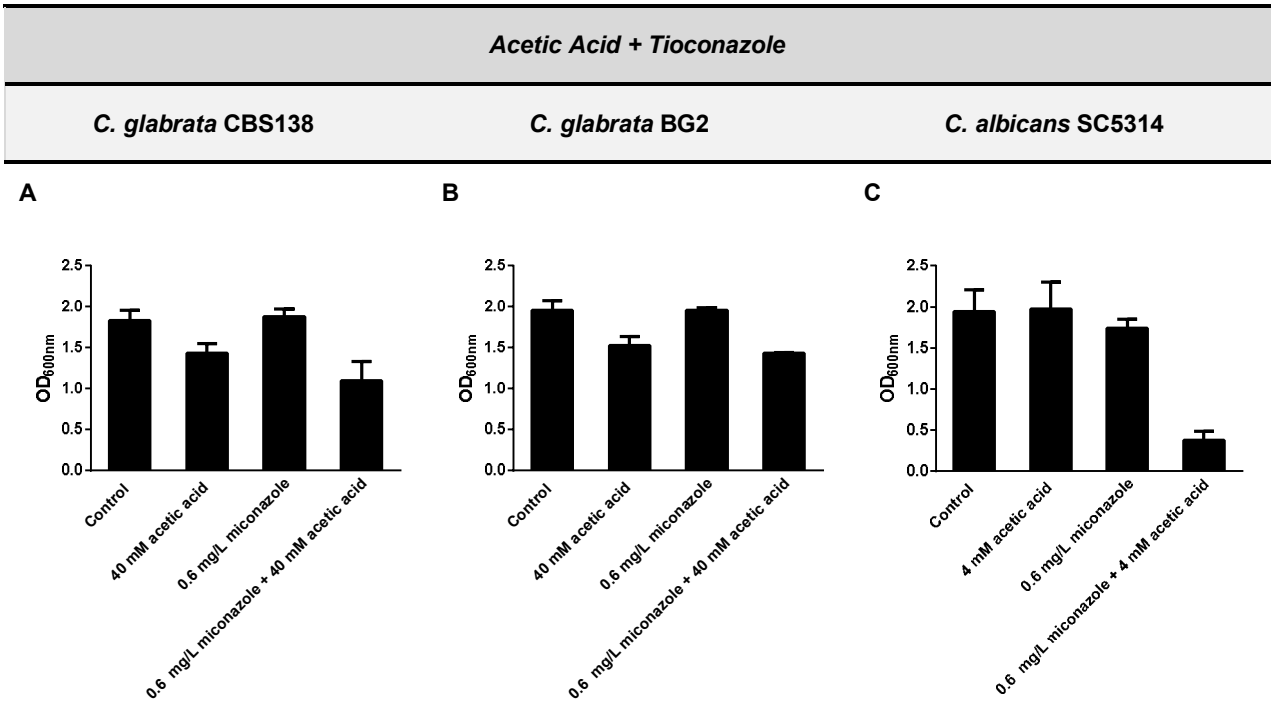


Figure 21 - Graphic representation of the ODs obtained in the assays done to try to assess the potential synergistic effect of acetic acid with tioconazole after 24h of growing in MM 1% glucose at 200 rpm for *C. blabrata* CBS138 (A), *C. glabrata* BG2 (B) and C. albicans SC5314 (C)

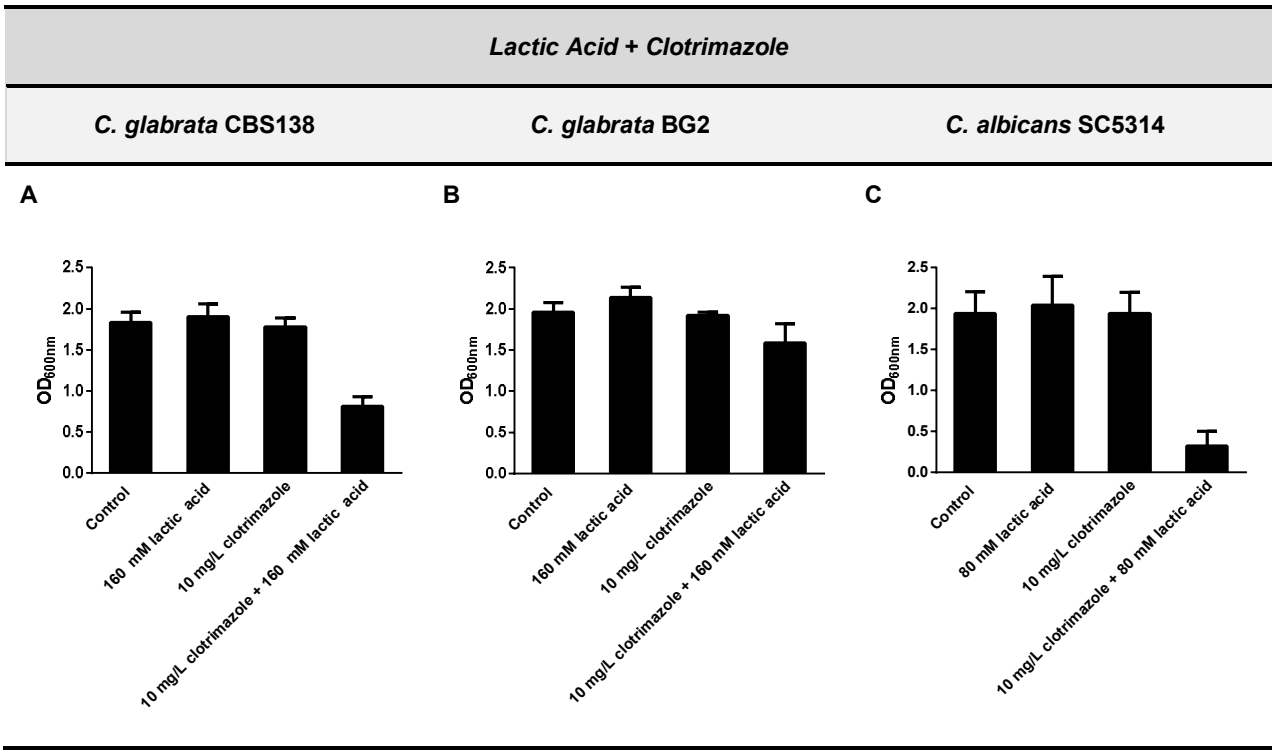


Figure 22 - Graphic representation of the ODs obtained in the assays done to try to assess the potential synergistic effect of lactic acid with clotrimazole after 24h of growing in MM 1% glucose at 200 rpm for *C. blabrata* CBS138 (A), *C. glabrata* BG2 (B) and C. albicans SC5314 (C).

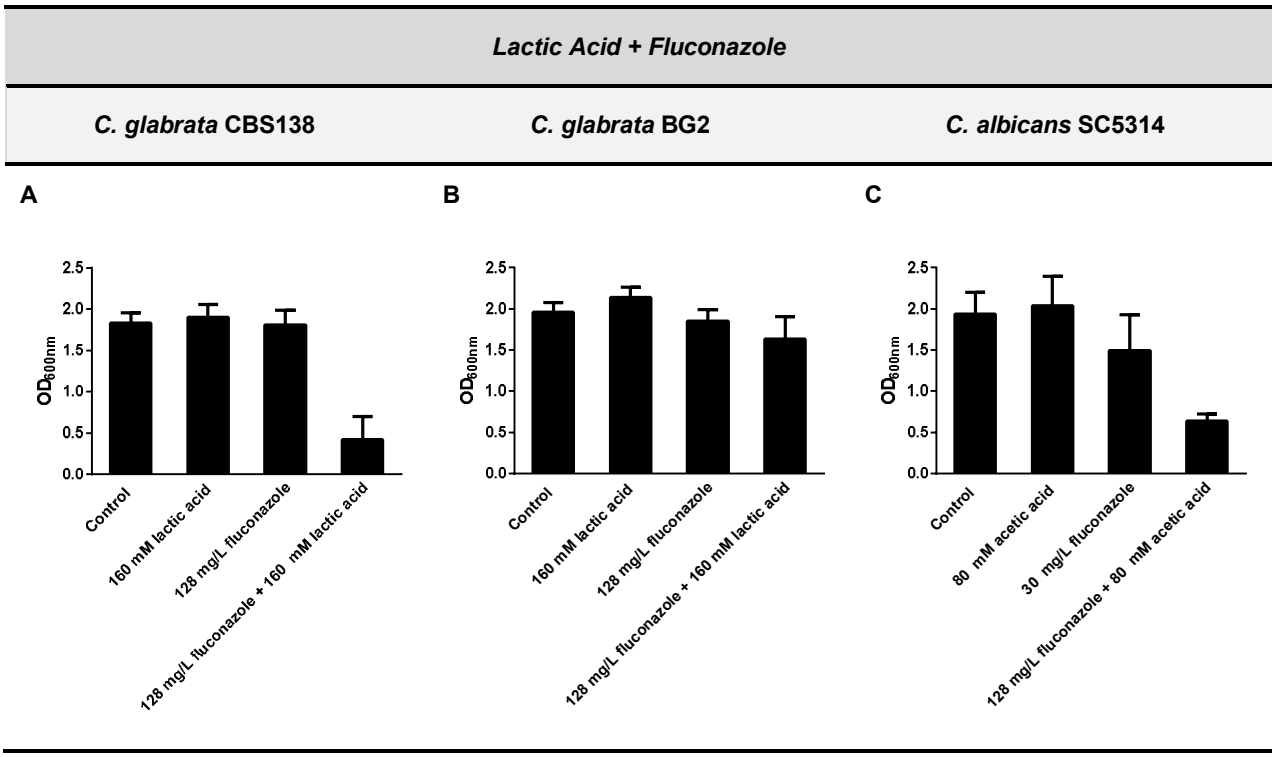


Figure 23 - Graphic representation of the ODs obtained in the assays done to try to assess the potential synergistic effect of lactic acid with fluconazole after 24h of growing in MM 1% glucose at 200 rpm for *C. blabrata* CBS138 (A), *C. glabrata* BG2 (B) and C. albicans SC5314 (C).

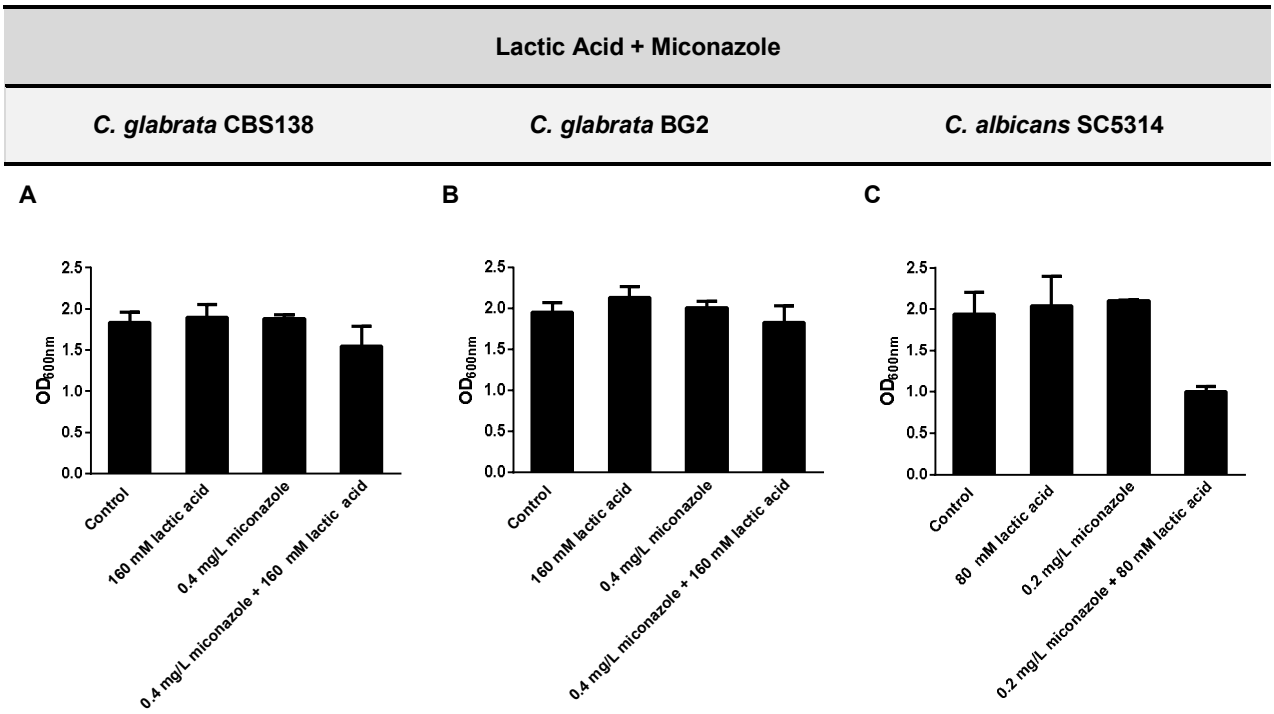


Figure 24 - Graphic representation of the ODs obtained in the assays done to try to assess the potential synergistic effect of lactic acid with miconazole after 24h of growing in MM 1% glucose at 200 rpm for *C. glabrata* CBS138 (A), *C. glabrata* BG2 (B) and C. albicans SC5314 (C).

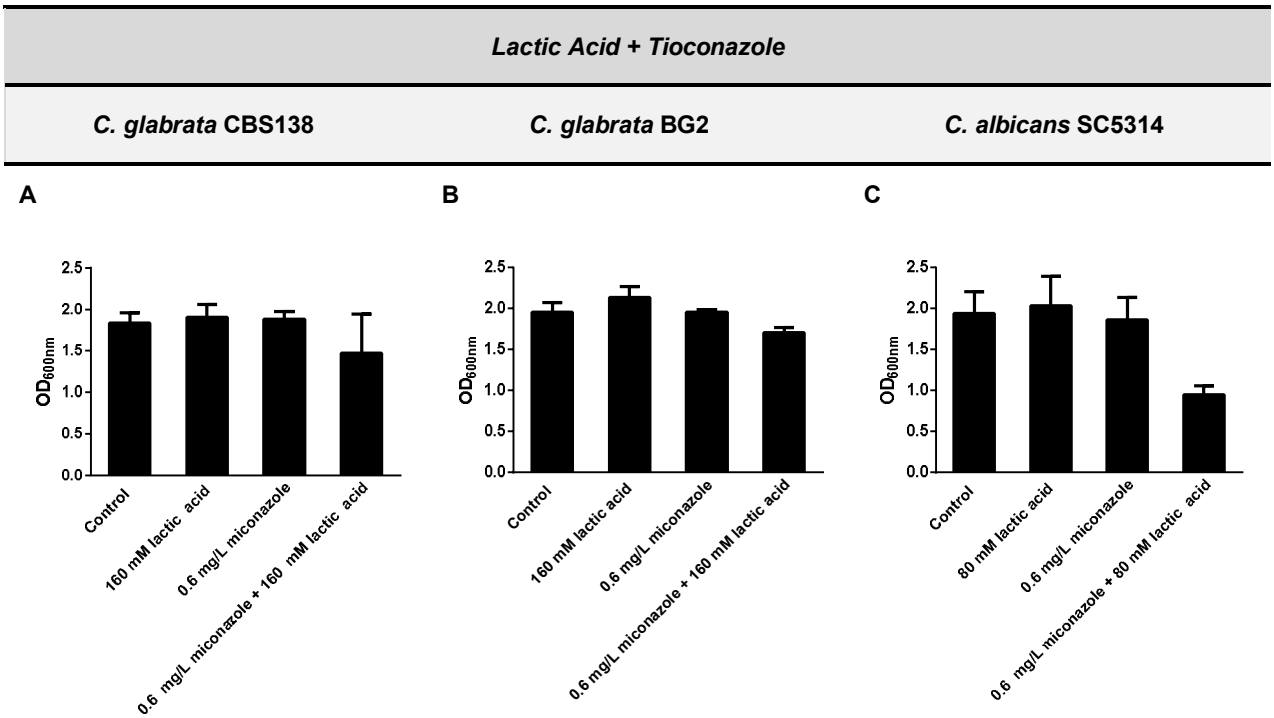


Figure 25 - Graphic representation of the ODs obtained in the assays done to try to assess the potential synergistic effect of lactic acid with miconazole after 24h of growing in MM 1% glucose at 200 rpm for *C. blabrata* CBS138 (A), *C. glabrata* BG2 (B) and C. albicans SC5314 (C).

3.4. 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 6 and 24h 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 (Figure 26). 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 26A and B). 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.

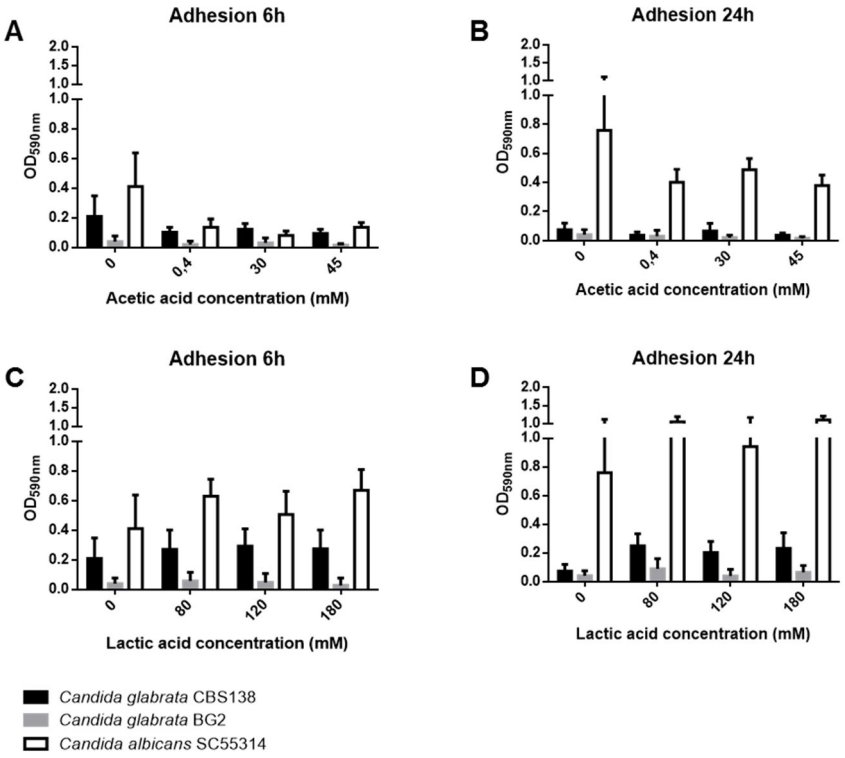


Figure 26 - Results obtained by crystal violet method to assess the effect of acetic and lactic acids in adhesion of *C. glabrata* CBS138, *C.glabrata* BG2 and *C. albicans* SC5314 to abiotic surface (polystyrene). Results obtained at 590nm after growing in MM 1% glucose at pH4 with acetic acid after 6h (A) and 24h (B) and with the supplementation with lactic acid after 6h (C) and 24h (D) of growing.

3.5. Functional analysis of the acetic acid-responsive genes Ad1, Ad2, Ad3 and Ad4 in *C. glabrata*

In the second part of this work it was performed the functional analysis of the *C. glabrata* genes *Ad1, Ad2, Ad3 and Ad4*. These poorly characterized adhesin-encoding genes were found to be transcriptionally activated in response to acetic acid by the CgHaa1 transcription factor (Bernardo et al. 2016). The functional analysis performed was focused on the involvement of these genes in tolerance to azoles, in adhesion to biotic and abiotic surfaces and in virulence against two infection models, *Galleria mellonella* and *Caenorhabditis elegans*.

3.5.1. 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 4). 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 4). This effect was augmented by the deletion of the *CgHAA1* gene (Table 4), 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. 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.

Table 4 - MICS of azoles of *C. glabrata* KUE100, obtained at normal pH, 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

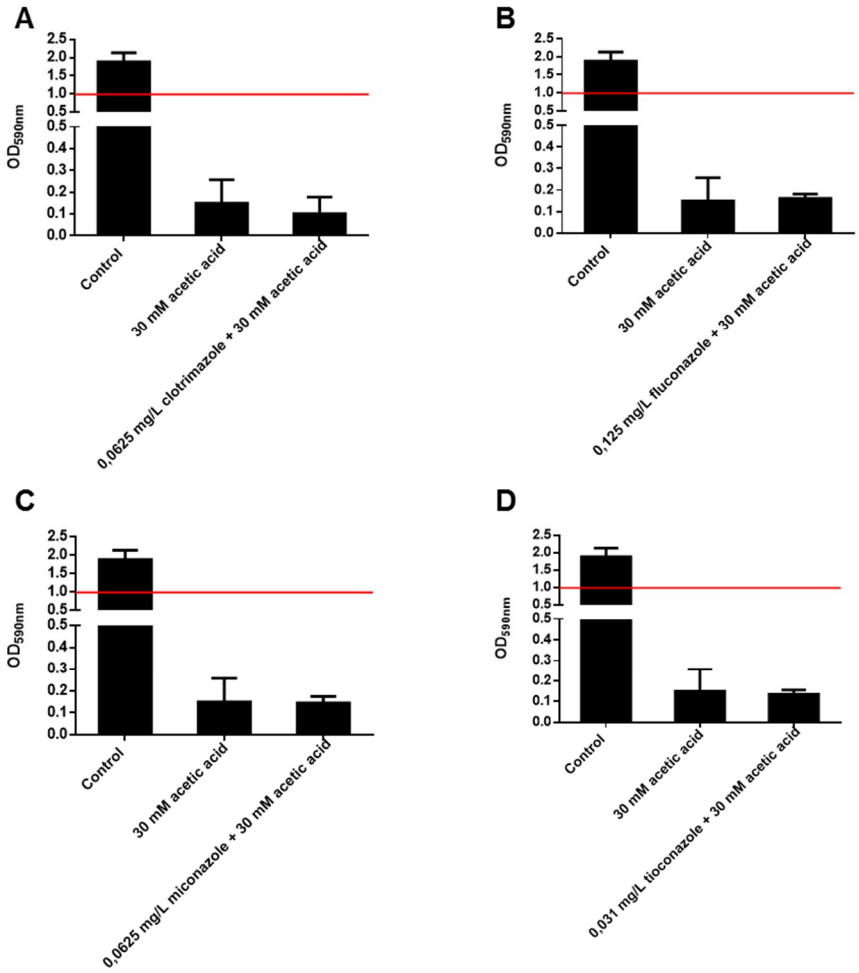
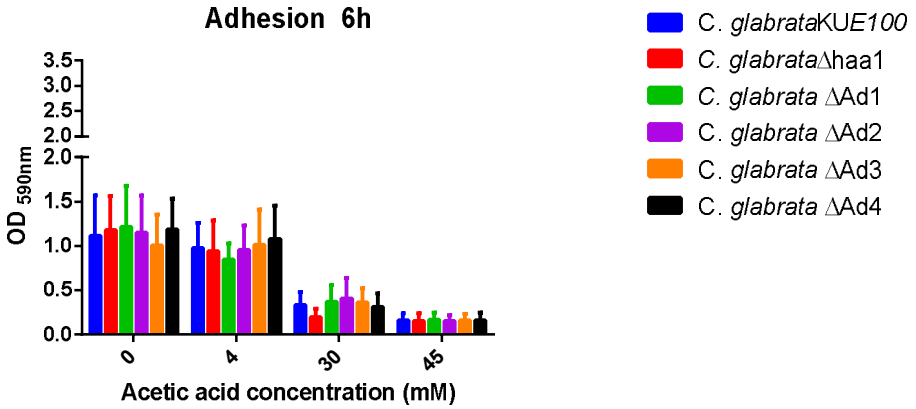


Figure 27 - Effect of acetic acid in the MIC concentrations for clotrimamazole (A), fluconazole (B); miconazole (C) and tioconazol (D) in *C. glabrata* ΔHaa1. Red line is the OD below which is assumed to be the MIC (half of the control OD).

3.5.2. 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 28), demonstrating that under these conditions the expression of the adhesins Ad1, Ad2, Ad3 and Ad4 is dispensable for biofilm formation.

A



B

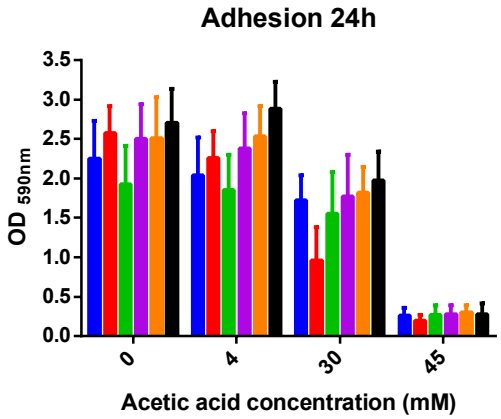


Figure 28 - Results obtained by crystal violet method to assess the effect of acetic acid in adhesion of *C. glabrata* strains to abiotic surface (polystyrene). Results obtained at 590nm after growing in RPMI 2% glucose with acetic acid after 6h (A) and 24h (B).

3.5.3. 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 29) 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.

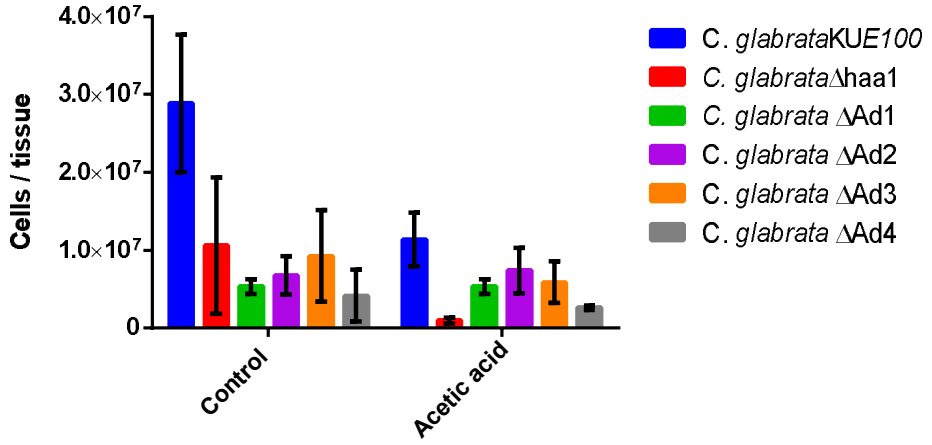


Figure 29 - Effect of acetic acid in cell adhesion of *C. glabrata* KUE100, ΔHaa1, ΔAd1, ΔAd2, ΔAd3 and ΔAd4 after 12 hours in RPMI 2% glucose at pH4

3.5.4. 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 30). 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 Ad2 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 30). The ΔAd4 was the *C. glabrata* mutant that registered the higher percentage of survival.

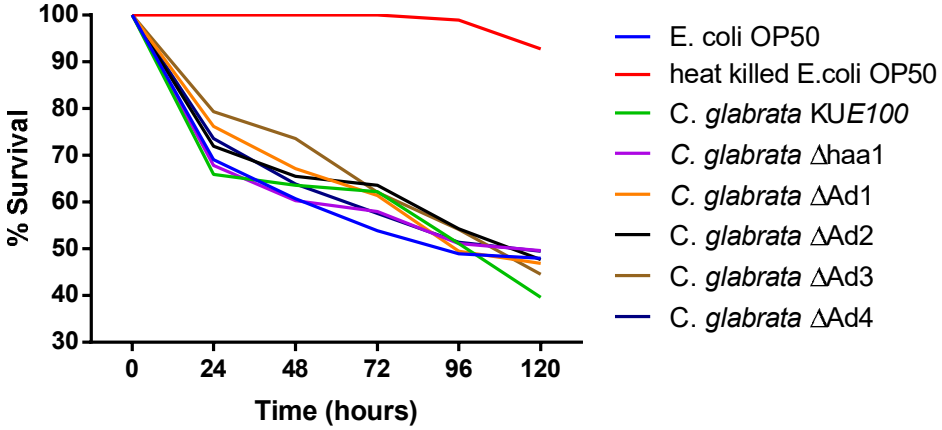


Figure 30 - Survival curves of *Caenorhabditis elegans* infected with *C. glabrata* KUE100 and *C. glabrata* Δhaa1 ΔAWP12, ΔAWP13, ΔAd3 and ΔAd4 (based on two replicates)

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 31. 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.

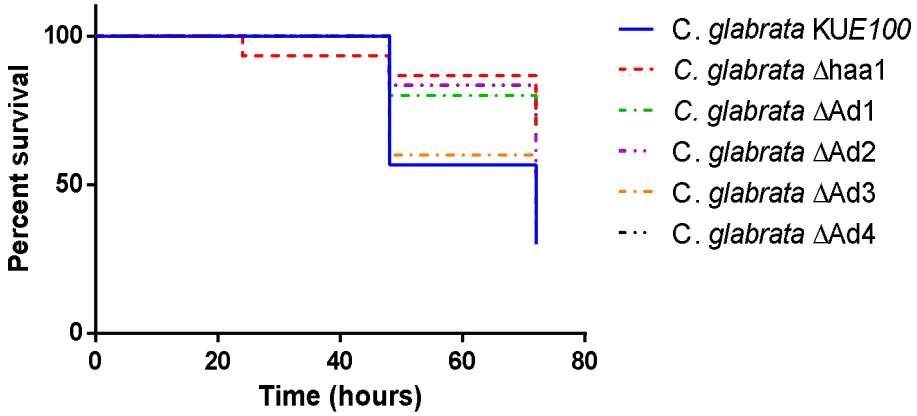


Figure 31- Kaplan–Meier survival curves of *Galleria mellonella* infected with *C. glabrata* KUE100, ΔCgHaa1, ΔAd1, ΔAd2, ΔAd3 and ΔAd4

4. 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 26 and Figure 28). 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.

5. References

Abbott, Derek A, Erwin Suir, Giang-huong Duong, Erik De Hulster, Jack T Pronk, and Antonius J A Van Maris. 2009. "Catalase Overexpression Reduces Lactic Acid-Induced Oxidative Stress in *Saccharomyces cerevisiae*", Applied And Environmental Microbiology, 75 (8): 2320–25.

Achkar, Jacqueline M, and Bettina C Fries. 2010. "*Candida* Infections of the Genitourinary Tract", Clinical Microbiology Reviews 23 (2): 253–73.

Ackerman, A Lenore, Jennifer T Anger, Karyn S Eilber, Vincent A Funari, Jie Tang, Jayoung Kim, and Michael R Freeman. 2016. "Identification Of A Diverse Fungal Community (``Mycobiome'') In The Normal Female Human Lower Urinary Tract." The Journal of Urology 195 (4).

Aldunate, Muriel, Daniela Srbinovski, Anna C Hearps, Catherine F Latham, Paul A Ramsland, Raffi Gugasyan, Richard A Cone, and Gilda Tachedjian. 2015. "Antimicrobial and Immune Modulatory Effects of Lactic Acid and Short Chain Fatty Acids Produced by Vaginal Microbiota Associated with Eubiosis and Bacterial Vaginosis", Frontiers in Physiology, 6 (164).

Aldunate, Muriel, David Tyssen, Adam Johnson, Tasnim Zakir, Secondo Sonza, Thomas Moench, Richard Cone, and Gilda Tachedjian. 2013. "Vaginal Concentrations of Lactic Acid Potently Inactivate HIV,", Journal of Antimicrobial Chemotherapy, 68: 2015-2025

Alves, Carlos Tiago, Xiao-qing Wei, Joana Azeredo, and Mariana Henriques. 2014. "*Candida albicans* Promotes Invasion and Colonisation of *Candida glabrata* in a Reconstituted Human Vaginal Epithelium,", Journal of Infection, 69: 396–407.

Bauters, Tiene G M, D Pharm, Marc A Dhont, Marleen I L Temmerman, and Hans J Nelis. 2002. "Prevalence of Vulvovaginal Candidiasis and Susceptibility to Fluconazole in Women," Am J Obstet Gynecol, 569–574.

Beigi, Richard H, Leslie A Meyn, Donna M Moore, Marijane A Krohn, and Sharon L Hillier. 2004. "Vaginal Yeast Colonization in Nonpregnant Women : A Longitudinal Study", The American College of Obstetricians and Gynecologists, 104 (5): 926–30.

Bernardo, Ruben T., Diana V Cunha, Can Wang, Leonel Pereira, Sónia Silva, Sara B Salazar, Markus S Schröder, et al. 2016. "The *CgHaa1*-Regulon Mediates Response and Tolerance to Acetic Acid Stress in the Human Pathogen *Candida glabrata*." G3: Genes, Genomes and Genetics; 7: 1-14.

Berterame, Nadia Maria, Danilo Porro, Diletta Ami, and Paola Branduardi. 2016. "Protein Aggregation and Membrane Lipid Modifications under Lactic Acid Stress in Wild Type and OPI1 Deleted *Saccharomyces cerevisiae* Strains." Microbial Cell Factories. 15 (39): 1–12.

Borges, Sandra, Joana Silva, and Paula Teixeira. 2013. "The Role of Lactobacilli and Probiotics in Maintaining Vaginal Health.", Archives of Gynecology and Obstetrics.

Boskey, E R, K M Telsch, K J Whaley, and T R Moench. 1999. "Acid Production by Vaginal Flora In Vitro Is Consistent with the Rate and Extent of Vaginal Acidification", Infection And Immunity, 67 (10): 5170–5175.

Brunke, Sascha, and Bernhard Hube. 2013. "Microreview Two Unlike Cousins : *Candida albicans* and

C . glabrata Infection Strategies", Cellular Microbiology, 15(5), 701–708.

Chen, Yingying, Lisa Stabryla, and Na Wei. 2016. "Improved Acetic Acid Resistance in *Saccharomyces cerevisiae* by Overexpression of the WHI2 Gene Identified through Inverse", Applied and Environmental Microbiology; 82 (7): 2156–2166.

Childers, Delma S, Ingrida Raziunaite, Gabriela Mol Avelar, Joanna Mackie, Susan Budge, M Maccallum, and Alistair J P Brown. 2016. "The Rewiring of Ubiquitination Targets in a Pathogenic Yeast Promotes Metabolic Flexibility , Host Colonization and Virulence", PLOS Pathogens, 1–26.

Cottier, Fabien, Alrina Shin, Min Tan, Jinmiao Chen, Josephine Lum, Francesca Zolezzi, Michael Poidinger, and Norman Pavelka. 2015. "The Transcriptional Stress Response of *Candida albicans* to Weak Organic Acids", G3: Genes, Genomes and Genetics , 5: 497–505.

Cunha, Diana Varandas -" Molecular mechanisms underlying tolerance to acetic acid in vaginal *Candida glabrat*a clinical isolates: role of the *CgHaa1* dependent system", Lisboa: nstituto Superior Técnico - Universidade de Lisboa, 2014, Tese de Mestrado.

Doerflinger, Sylvie Y., Andrea L Throop, and Melissa M Herbst-kralovetz. 2014. "Bacteria in the Vaginal Microbiome Alter the Innate Immune Response and Barrier Properties of the Human Vaginal Epithelia in a Species- Specific Manner", The Journal of Infectious Desiases, 209: 1989–99.

Dupuy, Amanda K, Marika S David, Lu Li, Thomas N Heider, Jason D Peterson, Elizabeth A Montano, Anna Dongari-bagtzoglou, Patricia I Diaz, and Linda D Strausbaugh. 2014. "Redefining the Human Oral Mycobiome with Improved Practices in Amplicon-Based Taxonomy : Discovery of *Malassezia* as a Prominent Commensal", PLOS ONE, 9 (3): 1–11.

Lastauskienė, Eglė, Zinkevičienė A, Girkontaitė I, Kaunietis A, and Kvedarienė V. 2014. "Formic Acid and Acetic Acid Induce a Programmed Cell Death in Pathogenic *Candida* Species.", Current Microbiology.

Ene, Iuliana V, Shih-chin Cheng, Mihai G Netea, and Alistair J P Brown. 2013. "Growth of *Candida albicans* Cells on the Physiologically Relevant Carbon Source Lactate Affects Their Recognition and Phagocytosis by Immune Cells", Infection and Immunity, 81 (1): 238–248.

Foxman, Betsy, Ryan Muraglia, Jean-paul Dietz, Jack D Sobel, and Julian Wagner. 2013. "Prevalence of Recurrent Vulvovaginal Candidiasis in 5 European Countries and the United States : Results From an Internet Panel Survey.", Journal of Lower Genital Tract Disease, 17(3): 340-345.

Gonçalves, Bruna, Carina Ferreira, Carlos Tiago Alves, Mariana Henriques, Joana Azeredo, Sónia Silva, Bruna Gonçalves, et al. 2015. "Vulvovaginal Candidiasis: Epidemiology, Microbiology and Risk Factors." *Critical Reviews in Microbiology*, 1–23.

Gregori, Christa, Christoph Schu, Andreas Roetzer, Tobias Schwarzmu, Gustav Ammerer, and Karl Kuchler. 2007. "The High-Osmolarity Glycerol Response Pathway in the Human Fungal Pathogen *Candida glabrata* Strain ATCC 2001 Lacks a Signaling Branch That Operates in Baker ' S Yeast ", Eukaryotic Cell, 6 (9): 1635–1645.

O'Hanlon , Deirdre E., Thomas R Moench, and Richard A Cone. 2013. "Vaginal pH and Microbicidal Lactic Acid When Lactobacilli Dominate the Microbiota", PLOS ONE, 8 (11): 1–8.

Hasan, Fahmi, Immaculata Xess, Xiabo Wang, Neena Jain, and Bettina C. Fries. 2009. “Biofilm Formation in Clinical *Candida* Isolates and Its Association with Virulence”, Microbes Infect, 11(8-9): 753–761.

Hickey, Roxana J., Xia Zhou, Jacob D. Pierson, Jacques Ravel, and Larry J. Forney. 2013. “Understanding Vaginal Microbiome Complexity from an Ecological Perspective.” Transl Res, 160 (4): 267–82.

Huang, Ruijie, Mingyun Li, and Richard L Gregory. 2011. “Bacterial Interactions in Dental Biofilm,”, Landes Bioscience - Taylor & Francis Group, Virulence 2 (5), 435-444: 435–444.

Ilkit, Macit, and Ahmet Baris Guzel. 2011. “The Epidemiology , Pathogenesis , and Diagnosis of Vulvovaginal Candidosis : A Mycological Perspective The Epidemiology , Pathogenesis , and Diagnosis of Vulvovaginal Candidosis : A Mycological Perspective”, Critical Reviews in Microbiology; 37 (3): 250–261.

Jeavons, Heather S. 2003. “Prevention and Treatment of Vulvovaginal Candidiasis Using Exogenous Lactobacillus.” *Journal of Obstetric, Gynecologic, & Neonatal Nursing*; 32: 287–96.

Kabir, M Anaul, Mohammad Asif Hussain, and Zulfiqar Ahmad. 2012. “*Candida albicans* : A Model Organism for Studying Fungal Pathogens” , ISRN Microbiology, 1-15.

Koszul, Romain, Alain Malpertuy, Lionel Frangeul, Christiane Bouchier, Patrick Wincker, Christophe Hennequin, and Bernard Dujon. 2003. “The Complete Mitochondrial Genome Sequence of the Pathogenic Yeast *Candida* (*torulopsis*) *glabrata*”, FEBS Letters 534:39-48.

Krause, Robert, Bettina Halwachs, Gerhard G Thallinger, Ingeborg Klymiuk, Katharina Heidrich, Walter Buzina, Helmut J F Salzer, and Jasmin Rabensteiner. 2016. “Characterisation of *Candida* within the Mycobiome / Microbiome of the Lower Respiratory Tract of ICU Patients,”, PLOS ONE, 1–29.

Kucharíková. 2016. “Detailed Comparison of *Candida albicans* and *Candida glabrata* Biofilms under Different Conditions and Their Susceptibility to Caspofungin and Anidulafungin,”, Journal of Medical Microbiology, 60:1261–1269.

Mcdonald, L C, R F Mcfeeters, M A Daeschel, H P Fleming, North Carolina, North Carolina, and North Carolina. 1987. "A Differential Medium for the Enumeration of Homofermentative and Heterofermentative Lactic Acid Bacteriat”, Applied And Environmental Microbiology, 53 (6): 1382–1384.

Merhej, Jawad, Antonin Thiebaut, Corinne Blugeon, Juliette Pouch, Mohammed El, Amine Ali, Jean-michel Camadro, and Stéphane Le Crom. 2016. “A Network of Paralogous Stress Response Transcription Factors in the Human Pathogen *Candida glabrata*”, Frontiers in Microbiology, 7: 1–16.

Mil-Homens, D., S. Ferreira-Dias, and A. M. Fialho. 2016. “Fish Oils against *Burkholderia* and *Pseudomonas aeruginosa* : In Vitro Efficacy and Their Therapeutic and Prophylactic Effects on Infected *Galleria mellonella* Larvae,”, Journal of Applied Microbiology, 1–11.

Mintz, Jason D, and Mark G Martens. 2013. “Prevalence of Non- *albicans Candida* Infections in Women with Recurrent Vulvovaginal Symptomatology”, Advances in Infectious Diseases, 3: 238-242.

Mira, Nuno P, Jorg D Becker, and Isabel Sa. 2010. “Genomic Expression Program Involving the Haa1p-Regulon in *Saccharomyces cerevisiae* Response to Acetic Acid”, OMICS A Journal of Integrative Biology, 14 (5): 587-601.

Mira, Nuno P, F Henriques, Greg Keller, Miguel C Teixeira, M Arraiano, Dennis R Winge, Isabel Sa, and G Matos. 2011. “Identification of a DNA-Binding Site for the Transcription Factor Haa1 , Required for *Saccharomyces cerevisiae* Response to Acetic Acid Stress”, Nucleic Acids Research, 39 (16): 6896–6907.

Mira, Nuno P, Miguel Cacho Teixeira, and Isabel Sa. 2010. “Adaptive Response and Tolerance to Weak Acids in *Saccharomyces cerevisiae* : A Genome-Wide View”, OMICS: A Journal of Integrative Biology, 14 (5): 525-540.

Mollapour, Mehdi, Andy Shepherd, and Peter W Piper. 2008. “Novel Stress Responses Facilitate *Saccharomyces cerevisiae* Growth in the Presence of the Monocarboxylate Preservatives,”, Yeast - Wiley Online Library, February: 169–77.

Moosa, Mahomed-yunus S, Jack D Sobel, Hussain Elhalis, Wenjin Du, and Robert A Akins. 2004. “Fungicidal Activity of Fluconazole against *Candida albicans* in a Synthetic Vagina-Simulative Medium”, Antimicrobial Agents And Chemotherapy,48 (1): 161–67.

Morales, Diana K., and Deborah A. Hogan. 2010. “*Candida* Albicans Interactions with Bacteria in the Context of Human Health and Disease.” *PLoS Pathogens* 6 (4): e1000886.

Mundy, Renee Domergue, and Brendan Cormack. 2009. “Expression of *Candida glabrata* Adhesins Following Exposure to Chemical Preservatives”, J Infect Dis., 199(12): 1891–1898.

Nagashima, Masahito, Yuka Yamagishi, and Hiroshige Mikamo. 2015. “Antifungal Susceptibilities of *Candida* Species Isolated from the Patients with Vaginal Candidiasis.” Journal of Infection and Chemotherapy. 2014–2016.

Nash, Evelyn E, Brian M Peters, Elizabeth A Lilly, Mairi C Noverr, and Paul L Fidel. 2016. “A Murine Model of *Candida glabrata* Vaginitis Shows No Evidence of an Inflammatory Immunopathogenic Response,” PLOS ONE, 1–14.

Nevitt, Tracy, and Dennis J Thiele. 2011. “Host Iron Withholding Demands Siderophore Utilization for *Candida Glabrata* to Survive Macrophage Killing”, PLoS Pathogens, 7 (3).1-15.

Owen, Derek H, and David F Katz. 1999. “A Vaginal Fluid Simulant” , Elsevier- Contraception, 59: 91–95.

Parolin, Carola, Antonella Marangoni, Luca Laghi, Claudio Foschi, Alberto Ñahui Palomino, Natalia Calonghi, Roberto Cevenini, and Beatrice Vitali. 2015. “Isolation of Vaginal Lactobacilli and Characterization of Anti- *Candida* Activity,”, PLOS ONE, 1–17.

Peeters, Elke, Hans J Nelis, and Tom Coenye. 2008. “Comparison of Multiple Methods for Quantification of Microbial Biofilms Grown in Microtiter Plates”, Journal of Microbiological Methods 72: 157–165.

Piper, Peter, Claudia Ortiz Calderon, and Kostas Hatzixanthis. 2001. “Weak Acid Adaptation : The Stress Response That Confers Yeasts with Resistance to Organic Acid Food Preservatives,”, Microbiology, 147: 2635–2642.

Piper, Peter W. 2011. "*Chapter 4 - Resistance of Yeasts to Weak Organic Acid Food Preservatives*.

Advances in Applied Microbiology". 1st ed. Vol. 77. Elsevier Inc.

Pukkila-worley, Read, Frederick M Ausubel, and Eleftherios Mylonakis. 2011. "*Candida albicans* Infection of *Caenorhabditis elegans* Induces Antifungal Immune Defenses", PLoS Pathogens 7 (6): 1-13.

Pukkila-worley, Read, Anton Y Peleg, Emmanouil Tampakakis, and Eleftherios Mylonakis. 2009. "*Candida albicans* Hyphal Formation and Virulence Assessed Using a *Caenorhabditis elegans* Infection Model ", Eukaryotic Cell, 8 (11): 1750–58.

Ramos, Daniel González, Arthur R Gorter De Vries, Sietske S Grijseels, Margo C Van Berkum, Steve Swinnen, Marcel Van Den Broek, Elke Nevoigt, Jean Marc G Daran, Jack T Pronk, and Antonius J A Van Maris. 2016. "A New Laboratory Evolution Approach to Select for Constitutive Acetic Acid Tolerance in *Saccharomyces cerevisiae* and Identification of Causal Mutations." Biotechnology for Biofuels. 1–18.

Reid, Gregor, Jessica A. Younes, Henny C. Van der Mei, Gregory B. Gloor, Rob Knight, and Henk J. Busscher. 2011. "Microbiota Restoration: Natural and Supplemented Recovery of Human Microbial Communities." Nature Reviews Microbiology 9 (1): 27–38.

Rosa, Maria I, R Silva, R Silva, C Silva, Bruno R Silva, L Souza, Kristian Madeira, Ana P Panatto, and Lidia R Medeiros. 2013. "Weekly Fluconazole Therapy for Recurrent Vulvovaginal Candidiasis : A Systematic Review and Meta-Analysis." European Journal of Obstetrics & Gynecology and Reproductive Biology, 167: 132–36.

Santos, Rui Filipe Ramos - "Role of the Candida glabrata drug: H^+ antiporters in biofilm formation and virulence: emphasis on *CgDTR1, CgQDR2, CgTPO1_2* and *CgTPO4",* Lisboa: Instituto Superior Técnico - Universidade de Lisboa, 2015, Tese de Mestrado.

Silva, Sónia, Melyssa Negri, Mariana Henriques, Rosário Oliveira, David W. Williams, and Joana Azeredo. 2011. "*Candida glabrata ,Candida parapsilosis* and *Candida tropicalis: biology*,epidemiology, pathogenicity and antifungal resistance", FEMS Microbiology Reviews, 36: 288–305

Stratford, Malcolm, Gerhard Nebe-von-caron, Hazel Steels, Michaela Novodvorska, Joerg Ueckert, and David B Archer. 2013. "International Journal of Food Microbiology Weak-Acid Preservatives : pH and Proton Movements in the Yeast *Saccharomyces cerevisiae*." International Journal of Food Microbiology, 161 (3): 164–71.

Wong, Sze Sarah Wah, Richard Yi, Tsun Kao, Kwok Yong Yuen, Yu Wang, Dan Yang, Perera Samaranayake, and Chaminda Jayampath Seneviratne. 2014. "In Vitro and In Vivo Activity of a Novel Antifungal Small Molecule against *Candida* Infections", PLOS ONE, 9 (1): 1-17.

Tang, Jie, Iliyan D Iliev, Jordan Brown, David M Underhill, and Vincent A Funari. 2015. "Mycobiome : Approaches to Analysis of Intestinal Fungi." Journal of Immunological Methods, 9 (1): 1–10.

Trček, Janja, Nuno Pereira Mira, and Laura R Jarboe. 2015. "Adaptation and Tolerance of Bacteria against Acetic Acid.", Applied Microbiology and Biotechnology.

Tscherner, Michael, Tobias Schwarzmüller, and Karl Kuchler. 2011. "Pathogenesis and Antifungal Drug Resistance of the Human Fungal Pathogen *Candida* Glabrata," Pharmaceuticals, 4: 169-186.

Ueno, Keigo, Yasuhiko Matsumoto, Jun Uno, Kaname Sasamoto, Kazuhisa Sekimizu, Yuki Kinjo, and Hiroji Chibana. 2011. “Intestinal Resident Yeast *Candida glabrata* Requires Cyb2p-Mediated Lactate Assimilation to Adapt in Mouse Intestine”, PLoS ONE, 6 (9): 1-13.

Ullah, Azmat, Gayathri Chandrasekaran, Stanley Brul, Gertien J Smits, Instituto Superior, and Instituto Superior. 2013. “Yeast Adaptation to Weak Acids Prevents Futile Energy Expenditure”, Frontiers in Microbiology, 4: 1–10.

Annex A

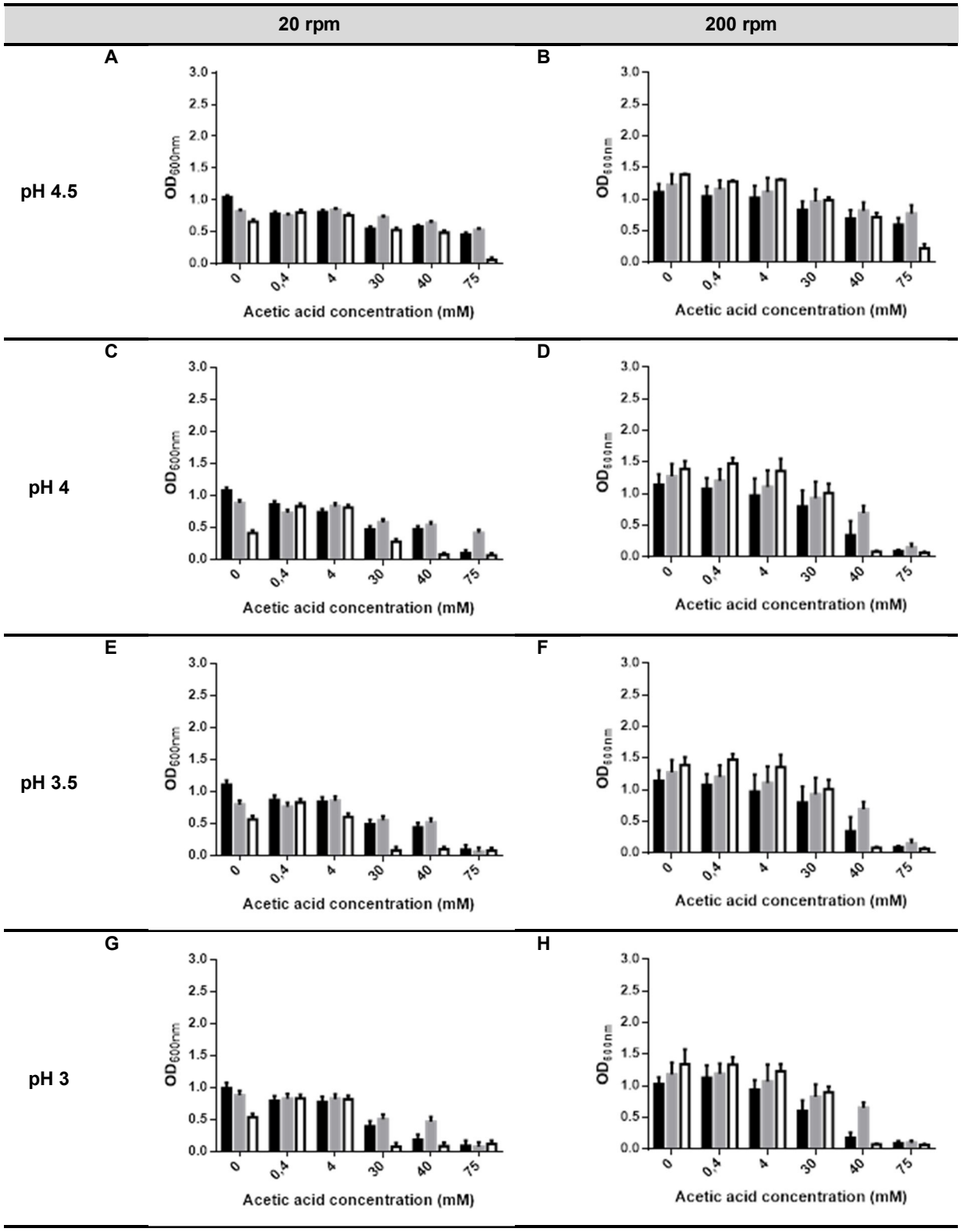


Figure S1 - Optical density at 600_{nm} of *C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314 after 24 hours of growth with acetic acid in RPMi 0,2% at 20 rpm at pH 4,5 (A), pH 4 (C), pH 3,5 (E), pH 3 (G) and at 200rpm at pH 4,5 (B), pH 4(D), pH 3.5 (F) and pH 3 (H).

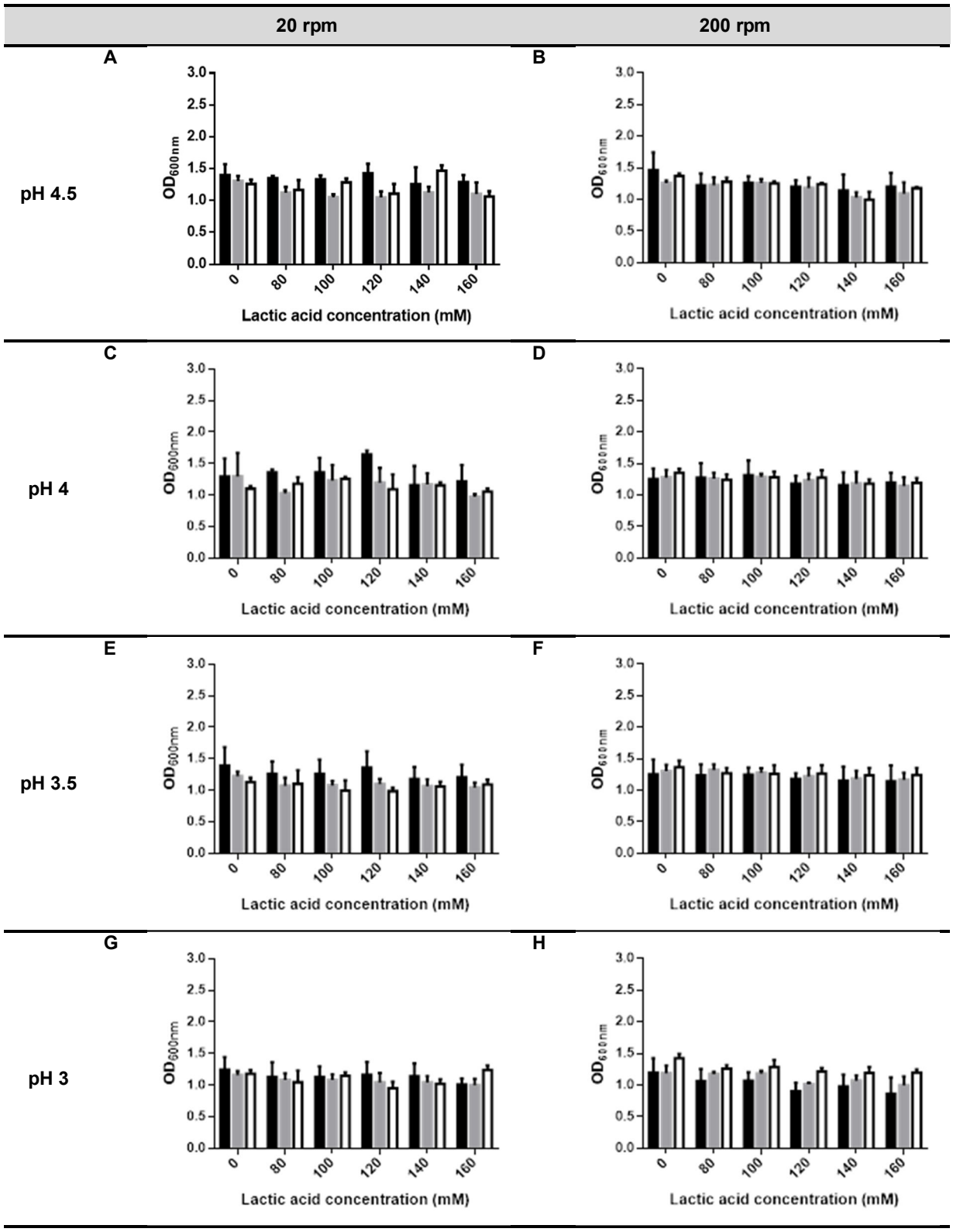


Figure S2 - Optical density at 600_{nm} of *C. glabrata* CBS138, *C. glabrata* BG2 and *C. albicans* SC5314 after 24 hours of growth with lactic acid in RPMi 0,2% at 20 rpm at pH 4,5 (A), pH 4 (C), pH 3,5 (E), pH 3 (G) and at 200rpm at pH 4,5 (B), pH 4(D), pH 3.5 (F) and pH 3 (H).

Annex B

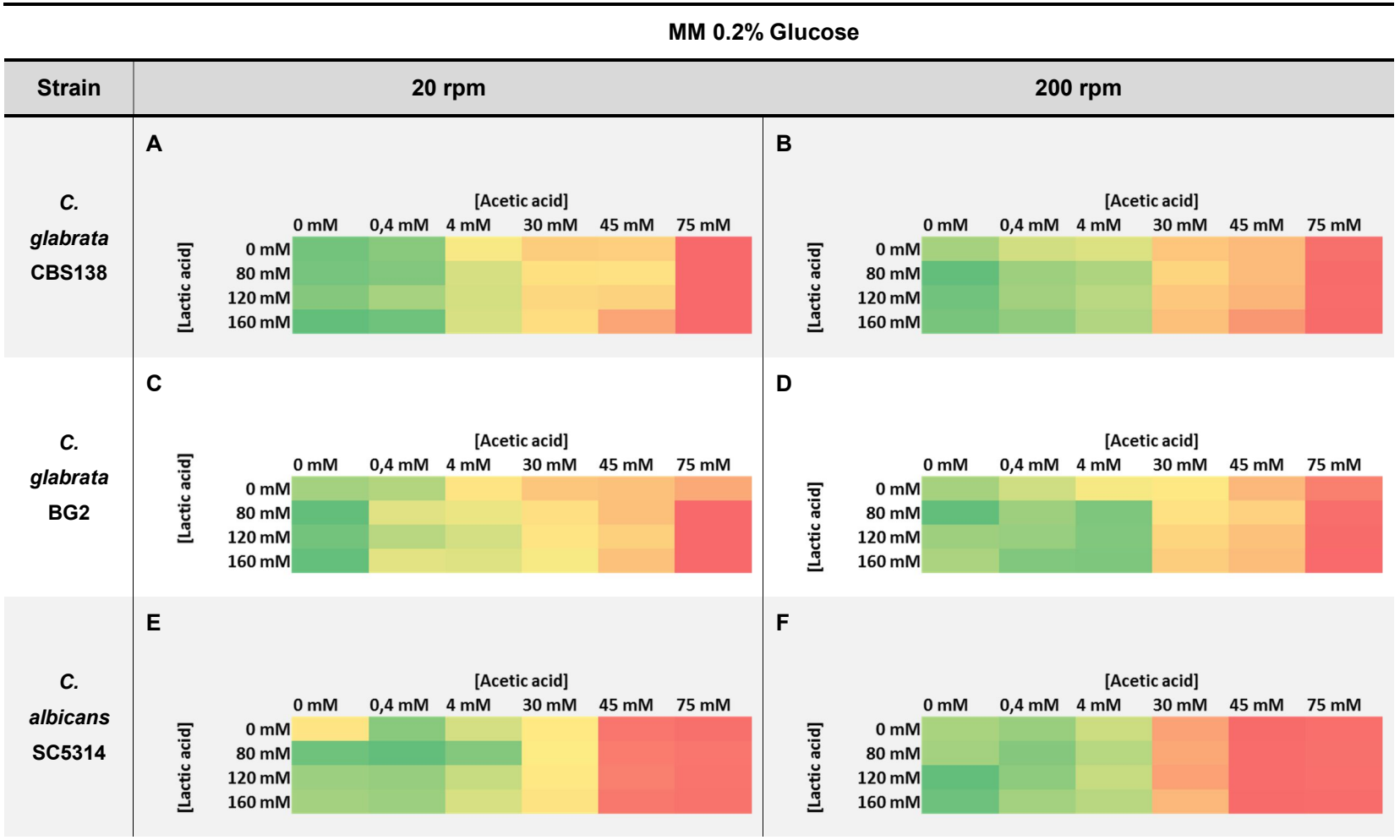


Figure S3 - Heatmaps obtained thought the assays to assess the potential synergistic effect of lactic and acetic acids in inhibition of *Candida* growth in MM 0.2% glucose at pH 4 after 24 hours at 20 rpm for *C. glabrata* CBS138 (A), *C. glabrata* BG2 (C) and *C. albicans* SC5314 (E) and at 200 rpm for *C. glabrata* CBS138 (B), *C. glabrata* BG2 (D) and *C. albicans* SC5314 (F). More dark green means more growth and more red more inhibition. Each heat map is formatted according it biggest value of OD.

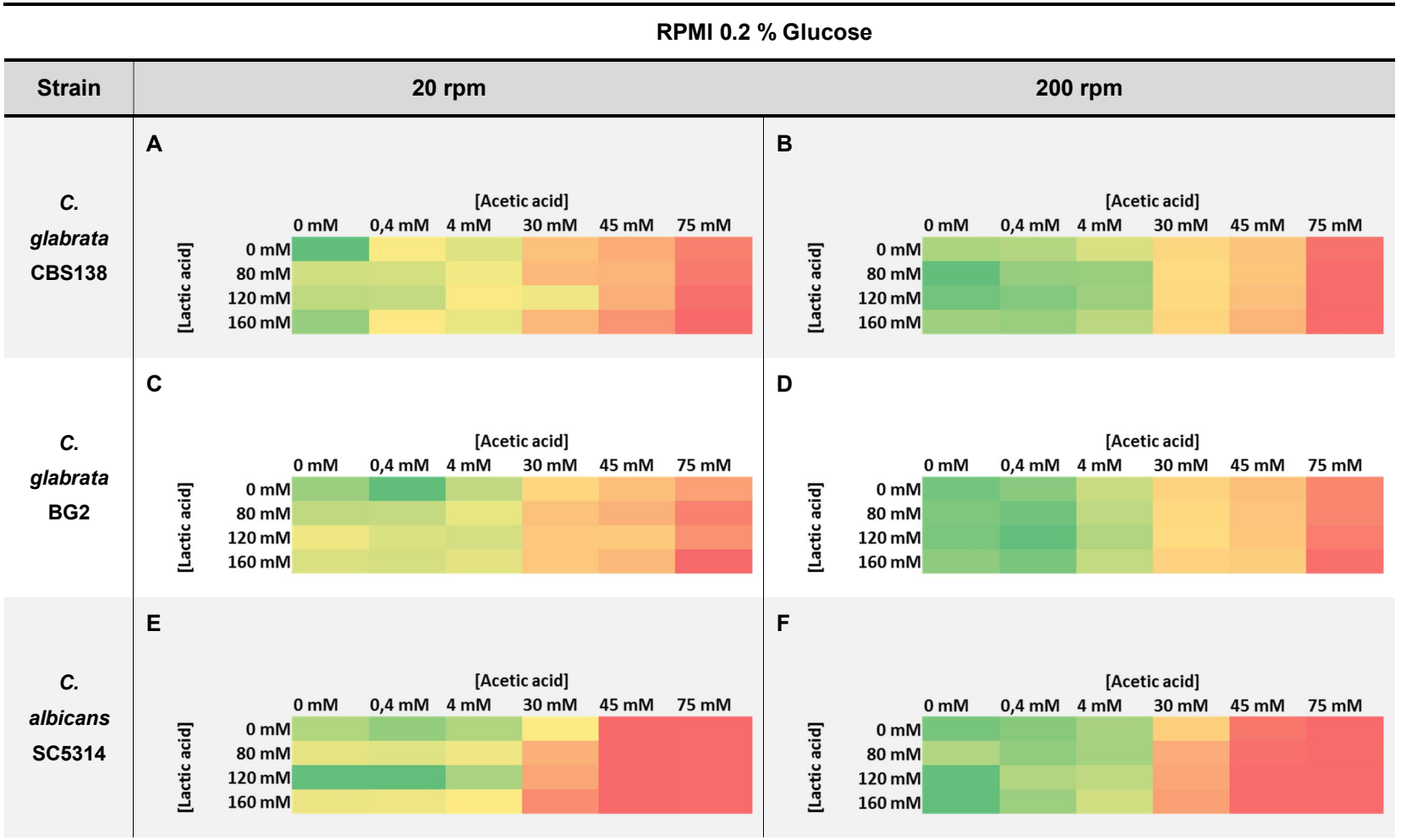


Figure S4 - Heatmaps obtained thought the assays to assess the potential synergistic effect of lactic and acetic acids in inhibition of *Candida* growth in RPMI 0.2% glucose at pH 4 after 24 hours at 20 rpm for *C. glabrata* CBS138 (A), *C. glabrata* BG2 (C) and *C. albicans* SC5314 (E) and at 200 rpm for *C. glabrata* CBS138 (B), *C. glabrata* BG2 (D) and *C. albicans* SC5314 (F). More dark green means more growth and more red more inhibition. Each heat map is formatted according it biggest value of OD