Physiological studies of co-cultivating *Candida albicans* or *Candida glabrata* in the presence of *Lactobacillus gasserii* or *Lactobacillus reuterii*

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Thesis to obtain the Master of Science Degree in

Microbiology

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Preface

The work presented in this thesis was performed at the Institute for Bioengineering and Biosciences of Instituto superior Técnico (Lisbon, Portugal), during the period September-October 2018-2019, under the supervision of Prof. Nuno Mira, and within the frame of the research project LactoCan - Fostering the development of new probiotic therapeutic approaches for the treatment of candidiasis through the exploration of lactobacilii-*Candida* interference mechanisms, funded by FCT-Portuguese Foundation for Science and Technology (contract PTDC/BIA-MIC/31515/2017).

Declaration

I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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Finally, my deepest gratitude goes to my parents in spite of being clueless of my work for most of the times, never failed to remind me of how far I've come and how proud they were of my accomplishments. Thank you for teaching me to be strong and never give up,

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Abstract

Candida species are part of the human commensal mycobiome, but also well-known causative agents of superficial and invasive infections generally known as candidiasis. In order to successfully colonize humans, *C. glabrata* and *C. albicans* have to face multiple environmental challenges including the presence of commensal bacterial microflora which has been found to have a strong interference effect. In this thesis it is further studied the interactions established between *C. albicans* and *C. glabrata* with the vaginal species *Lactobacillus gasseri* and the intestinal species *Lactobacillus reuteri*. Both *L. gasseri* and *L. reuteri* are shown to induce loss of viability of the two *Candida* species during co-cultivation, with a much stronger effect being observed for *L. reuteri* during co-cultivation in BHI medium. Consistently, supernatants obtained from *L. gasseri* and *L. reuteri* cultures in BHI and MRS medium showed a strong inhibitory effect in growth of *C. albicans* and *C. glabrata*, also affecting the ability of the first species to undergo filamentation, a critical virulence trait for this species. The results obtained do not support the idea that production of lactic acid underlies the observed inhibitory effect of the bacterial culture supernatants (or of the live bacterial cells), although the inhibitory effect was potentiated at acidic pHs. It is expected that the results obtained in this study might help to better understand the poorly characterized interference mechanisms between relevant lactobacilli species and *Candida*, thus fostering the development of new anti-*Candida* therapies.

Key - words: *Lactobacillus gasseri, Lactobacillus reuteri, Candida albicans, Candida glabrata, Lactobacillus* – *Candida* interference, supernatant.

Resumo

As espécies de *Candida* fazem parte do microbioma comensal humano, mas também são agentes causadores de infecções superficiais e invasivas, geralmente conhecidas como candidiase. A fim colonizar com sucesso os seres humanos, *C. glabrata* e *C. albicans* têm que enfrentar múltiplos desafios ambientais que incluem a presença da microflora comensal bacteriana, que é conhecida por ter um efeito marcante na interferência entre estes microrganismos. Nesta tese é estudado ainda as interações estabelecidas entre *C. albicans* e *C. glabrata* com a espécie vaginal *Lactobacillus gasseri* e a espécie intestinal *Lactobacillus reuteri*. Ambos *L. gasseri e L. reuteri* relatam induzir a perda de viabilidade das duas espécies *Candida* durante a co-cultura, com um efeito muito mais forte a ser observado para *L. reuteri* durante a co-cultura no meio BHI. Consistentemente, os sobrenadantes obtidos das culturas de *L. gasseri e L. reuteri* em BHI e no meio MRS mostraram um forte efeito inibitório no crescimento de *C. albicans e C. glabrata*, afetando também a capacidade da primeira espécie filamentar, uma caracteristica importante de virulência para esta espécie. Os resultados obtidos não suportam a ideia de que a produção de ácido láctico está subjacente ao efeito inibitório observado nos sobrenadantes da cultura bacteriana (ou das células bacterianas vivas), embora o efeito inibitório tenha sido potencializado em pHs ácidos. Espera-se que os resultados obtidos neste estudo possam ajudar a entender melhor os mecanismos de interferência mal caracterizados entre espécies de lactobacilii relevantes e *Candida*, promovendo assim o desenvolvimento de novas terapias anti-Candida.

Palavras-chave: *Lactobacillus gasseri, Lactobacillus reuteri, Candida albicans, Candida glabrata,* interação *Lactobacillus – Candida,* sobrenadante.

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Abbreviations

BV – bacterial vaginosis

Ca – *Candida albicans*

CFS – cell-free supernatant

CFU – Colony forming unit

Cg – *Candida glabrata*

EPS – exopolysaccharides

GIT – gastrointestinal tract

GUT – genitourinary tract

LA – lactic acid

LAB – lactic acid bacteria

MMB – Minimum Medium broth

MRS – De Man, Rogosa and Sharpe

OD – Optical Density

Rpm – Rotations per minute

RPMI – Roswell Park Memorial Institute medium

RVVC – Recurrent vulvovaginal candidiasis

VMB – vaginal microbiota

VMB – Vaginal Microbiota

VVC – vulvovaginal candidiasis

YPD – Yeast extract Peptone Dextrose

Chapter 1 – Introduction

1.1. Overview

The human body is composed of about 40 trillion human cells and about 22,000 human genes, however, as many as 10-100 trillion microbial cells (the microbiota) and 2 million microbial genes (the metagenome) are also present and cannot therefore be ignored (Martín *et al.*, 2014; Ravel *et al.*, 2014). Throughout all their life, humans are associated with microorganisms whose presence has been increasingly claimed to contribute positively for human health (Thomas *et al.*, 2017; Goltsman *et al.*, 2018; Pascale *et al.*, 2018). This cohort of microbial communities, generally known as the microbiome, consists of communities of commensal, symbiotic or pathogenic species that are distributed in different niches and that establish tight interactions, either among themselves but also with human cells (Brestoff and Artis, 2013). As can be seen by Figure 1, the presence of microbes is spread in the human body and it is composed by a huge variety of species, with the majority (around 70%) being located in the gastrointestinal tract (GIT). A noticeable feature is that the species found in the different sites do not necessarily coincide (as shown in Figure 1) probably reflecting the different adaptability of the species to the challenges posed by each environment. Species belonging to the *Lactobacillus* genus are an example of highly prevalent human colonizers being present in various niches including the genitourinary tract or the gastrointestinal tract, yet they are not found in the skin (Cui, Morris and Ghedin, 2013).

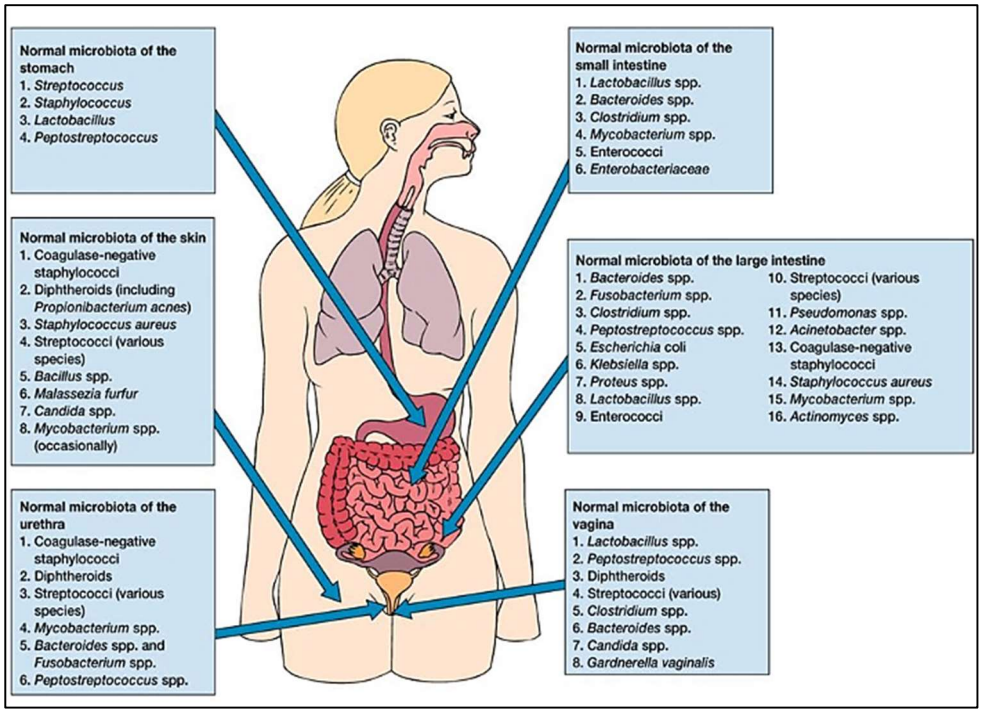

Figure 1 – Schematic representation of the distribution of microbial species present in different sites.

Although the term microbiome is usually associated with bacteria this is incorrect because humans are also colonized by a large number (although smaller than the bacterial ones) of fungal species giving rise to the so called concept of mycobiome (Cui, Morris and Ghedin, 2013; Bradford and Ravel, 2017). Through more recent species-profiling technologies based on metagenomics it has been determined that species of the *Candida* genus are very frequent fungal colonizers of humans being found in the genitourinary and gastrointestinal tracts, as well as in the skin and the oral cavity (Figure 1 and Figure 2) (Wheeler *et al.*, 2019). Notably, it has not been reported the presence of these species in the nasal cavity, for example (Cui, Morris and Ghedin, 2013).

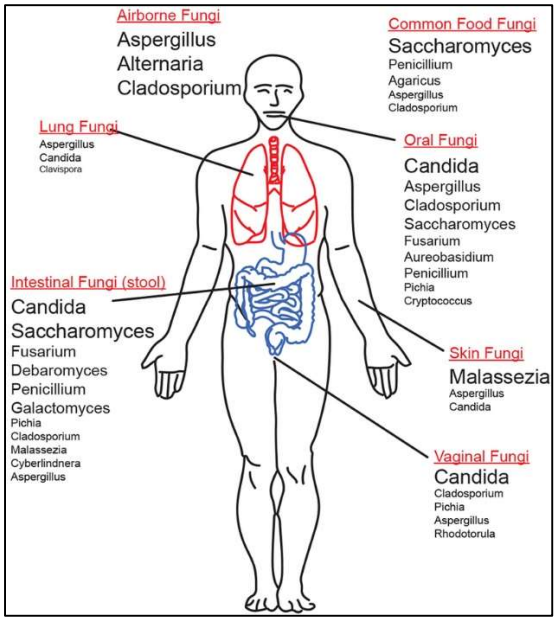

Figure 2 - Distribution of common commensal fungi. Fungal representation at various sites and sources are indicated and represented by the size of the fungal genera name (Wheeler *et al.*, 2019).

Necessarily, the co-habitation between micro/mycobiome and human cells leads to the establishment of interactions among these different players. These interactions can in addition be modulated by genetic factors of the host and/or by the environment (Figure 3). The outcome of these interactions may result in synergistic, mutualistic or antagonistic relations between the host and the microbial populations (Wang *et al.*, 2017).

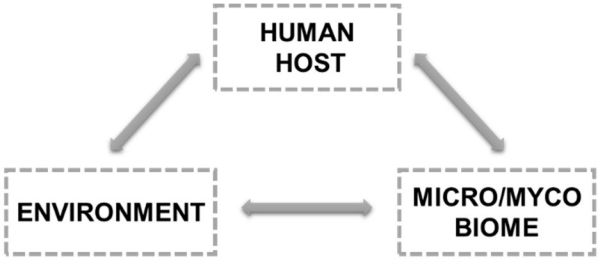

Figure 3 - The 3 open domains and their mutual interactions: ecosystem, bacterial communities and host.

The human vagina and the microbial communities that reside therein are a very good example of an excellently balanced mutualistic association (Figure 4 – left). For the benefit of the host, lactic-acid bacteria can prevent the overgrowth of pathogens, while in return the host provides the microbial communities the nutrients, and the habitat desired to support their growth. The continuous growth of the communities is vital since bacteria are continually shed from the body, through vaginal secretions (Ma, Forney and Ravel, 2012). As said above, in a healthy status (Figure 4 - left), the vaginal microbiome is largely dominated by *Lactobacilli*. When dysbiosis occurs, the vaginal microbiota turns into a polymicrobial community where *Lactobacilli* are no longer predominant (Figure 4 - right). This modification has been linked to an overgrowth of vaginal pathogens, including of *Candida* species, causing disease. Although some insights had been gathered concerning this inhibitory potential of *Lactobacilli* over pathogens (*Candida* species included), a thorough investigation on this interference mechanism has not been carried out, a knowledge that has the potential to contribute for the design of new therapeutic approaches by sensitizing *Candida* species through the improvement of *Lactobacilli* activity. This thesis is mainly focused on this field, aiming to study the effects of co-cultivation of the vaginal species *Lactobacillus gasseri* with *Candida albicans* or *Candida glabrata*. Because interference of *Lactobacilli* over growth of pathogens has also been shown to occur in the gastrointestinal tract (Suhr and Hallen-Adams, 2015), in this work we also examined how the intestinal species *L. reuterii* interacts with *C. albicans* and *C. glabrata*, this being based on the identification of these two yeasts as part of the commensal gastrointestinal microbiota. Remarkably, recent studies have been pointing to the relevance that commensal GI *Candida* populations have as a potential source for the occurrence of disseminated infections in the bloodstream (Lopez-Medina and Y. Koh, 2016; Tong and Tang, 2017; Pappas *et al.*, 2018; Wheeler *et al.*, 2019).

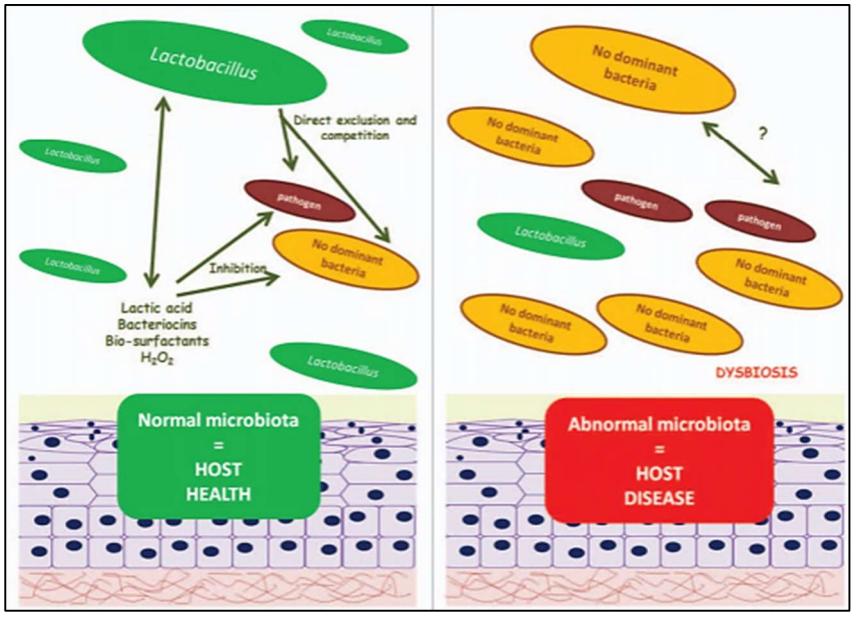

Figure 4 - Beneficial effect of lactobacilli on the vaginal ecosystem (Martín et al., 2014).

Brief overview on the positioning of *lactobacilli* and *Candida* spp. in the vaginal micro- and mycobiomes

The vagina is a complex ecosystem in a state of dynamic equilibrium and the gathering of data concerning its microbial equilibrium began over 150 years ago using light microscopy and figuring out the bacterial cultures that compose the vaginal microbiota - VMB (Martin, 2012). Along time microbiologists have demonstrated that vaginal bacterial communities of healthy women are dominated by species of lactic acid–producing bacteria (LAB), specially *Lactobacillus* (Figure 5) (Falagas, Betsi, & Athanasiou, 2006; Martín, Miquel, Langella, & Bermúdez-Humarán, 2014; Wang *et al*., 2017; Goltsman *et al*., 2018).

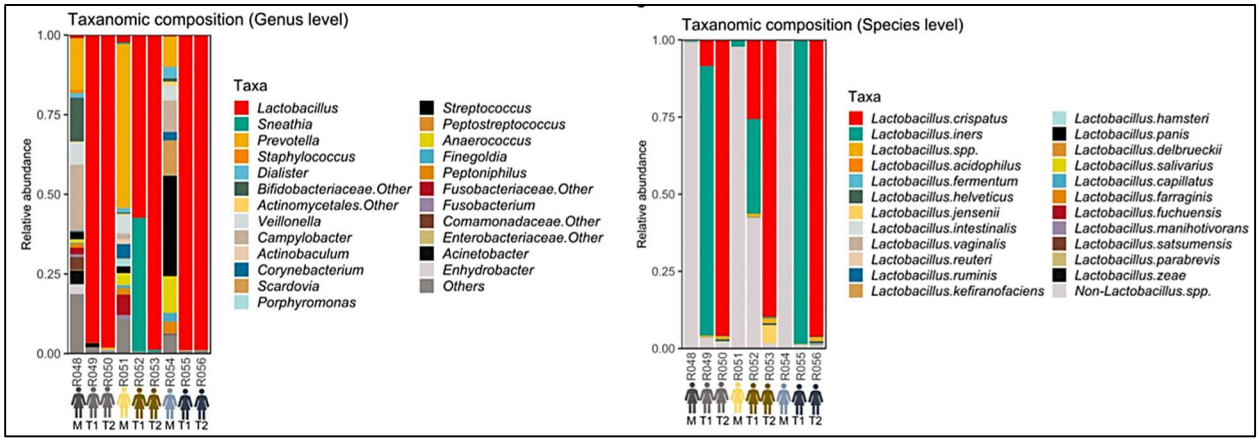

Figure 5 - Vaginal microbiota composition at the genus (left) and species (right) levels (Jang *et al.*, 2019).

In the last years, metagenomic analyses of vaginal samples have shown that bacterial communities are more complex than the expected. Based on 16S rRNA gene sequence it had been possible to analyze the vaginal community composition, as well as identify and perform a relative quantification of the bacterial taxa present. Metagenomic data reveal that the major genus found is *Lactobacillus* with the most common species including *L. crispatus, L. iners, L. jensenii, and L. gasseri* (Wang *et al.*, 2017; Goltsman *et al.*, 2018). The VMB is also habitat for other species as *Lactobacillus acidophilus* and *L. fermentum*, followed by *L. brevis*, *L. casei, L. gasseri, L. plantarum*, *L. fermentum, L. cellobiosus*, *L. brevis, L. minutus*, and *L. salivarius* (Segata *et al.*, 2013; Jang *et al.*, 2019). Less frequent vaginal colonizers include species of *Enterobacteriaceae*, *Acinetobacter* or *Enhydrobacter* genuses (Figure 5) (Falagas, Betsi and Athanasiou, 2006; Hickey *et al.*, 2012; Segata *et al.*, 2013; Jang *et al.*, 2019). Associations between the vaginal microbiota and race/ethnicity, age, or stage of life or lifestyle of women have also been made. For example, *Lactobacillus* predominate more the vaginal microbiome of Asian and white women (80,2% and 89,7% respectively) than black and Hispanic women (59,6% and 61,9% respectively) harboring Atopobium and a diverse array of phylotypes from the order Clostridiales, suggesting the existence of multiple "vagitypes". Overall, *L. iners* was the most common species of *Lactobacillus* in women of both ethnic groups (Zhou *et al.*, 2007; Hickey *et al.*, 2012; Fettweis *et al.*, 2013; Selle and Klaenhammer, 2013).

In this thesis it will be focused the role of *L. gasseri*, a dominant indigenous species of the vaginal tract in the modulation of growth and virulence of *C. albicans* and *C. glabrata*. *L. gasseri* presents itself as an anaerobic species, tolerant to acidic pHs and bile salts and to low CO_2 concentration (Lauer, E. & Kandler, 1980). This species is well documented by having a negative correlation with bacterial vaginosis and by conferring tolerance against vaginal pathogens. These traits render *L. gasseri* potential to serve as probiotic, leading to preliminary investigations for the purpose of clinical application and maintenance of GUT homeostasis (Selle and Klaenhammer, 2013).

While the bacterial composition of the vaginal niche has been extensively characterized, much less had been known on the mycobiota present therein, mainly due to limitations of the techniques used for this profiling which were largely based on culture (Figure 6). Metagenomics based on 18S rRNA sequencing identified 3 fungal phyla in vaginal samples: Ascomycota (22/28), of which *Candida* was the predominant genera, Basidiomycota (5/28) and Oomycota (1/28). At the species level it was found that the most abundant is *C. albicans*, followed by *C. glabrata,* both belonging to the predominant phylum Ascomycota (Bradford and Ravel, 2017).

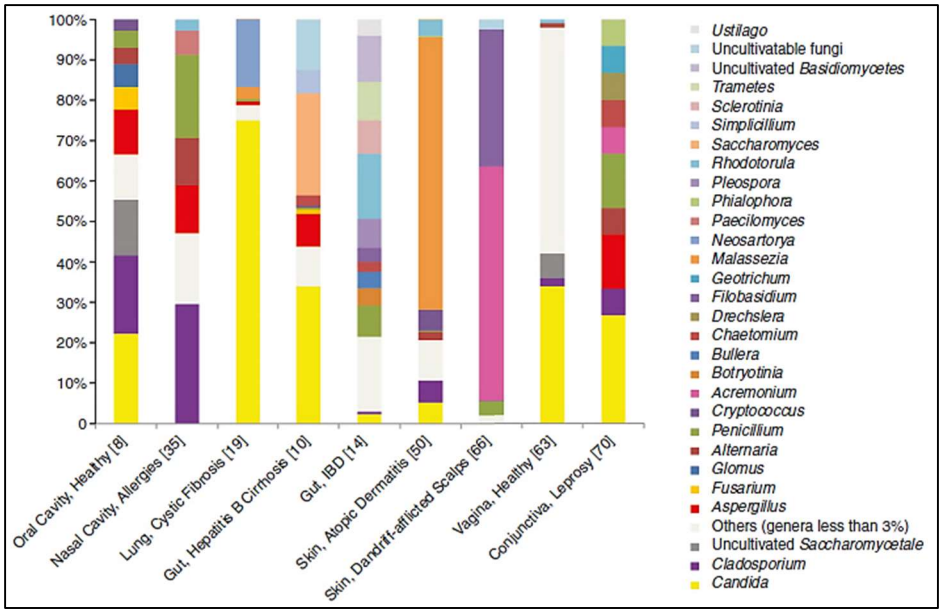

Figure 6 - Distribution of fungal genera in different body sites (Cui, Morris and Ghedin, 2013).

Virulence of *Candida* in the vaginal tract

The pathogenesis of *Candida* in the vaginal tract is believed to result from an overgrowth of the commensal population that may result for the development of a mucosal infection generally known as vaginal candidiasis. About 75% of all women are estimated to experience an (sporadic) episode of *Candida* vaginitis in their lifetime, with about 13% to 19% of VVC patients suffering from (frequent) repeated occurrences of this infection, a condition known as recurrent vulvovaginal candidiasis – RVVC (Foxman *et al.*, 2013). It was observed that vaginal microbiota in RVVC patients shown to be depleted from *lactobacilli*, what leads to a minor production of secondary metabolites with antimicrobial activity (Liang *et al.*, 2017). Some VVC patients also show a lack of H_2O_2 producing *Lactobacillus* species (Parolin *et al.*, 2015).

Among the key virulence factors known to play a role in vaginal virulence of *Candida spp*. are the ability of these yeasts of adhesion and formation of biofilms, the production of extracellular hydrolytic enzyme production, hyphal morphogenesis (reversible transitions between a form to disseminate and to adhering) and phenotypic switching (complementary response to different environments) (Silva *et al.*, 2009; Huang, 2012; Dantas *et al.*, 2016; Gonçalves *et al.*, 2016; Liang *et al.*, 2017; Wang *et al.*, 2017).

1.2. The lactobacilli-*Candida* interaction in the Genito-urinary tract

As said above, a reduction in the abundance of commensal populations has been associated with the development of various diseases including susceptibility to urogenital infections (Hickey *et al.*, 2012; Bradford and Ravel, 2017). A link between abundance of vaginal *lactobacilli* and the progress of *Candida* infections has been suggested by results of different studies including metagenomic analysis that revealed a decreased abundance of *Lactobacillus* in patients with VVC (Bradford and Ravel, 2017) although it also has to be mentioned that in some patients with VVC the abundance of lactobacilli population was identical to the one observed in control patients (Figure 7) (Liu *et al.*, 2013; Zhonghua Yi Xue Za Zhi., 2015; Pramanick *et al.*, 2019).

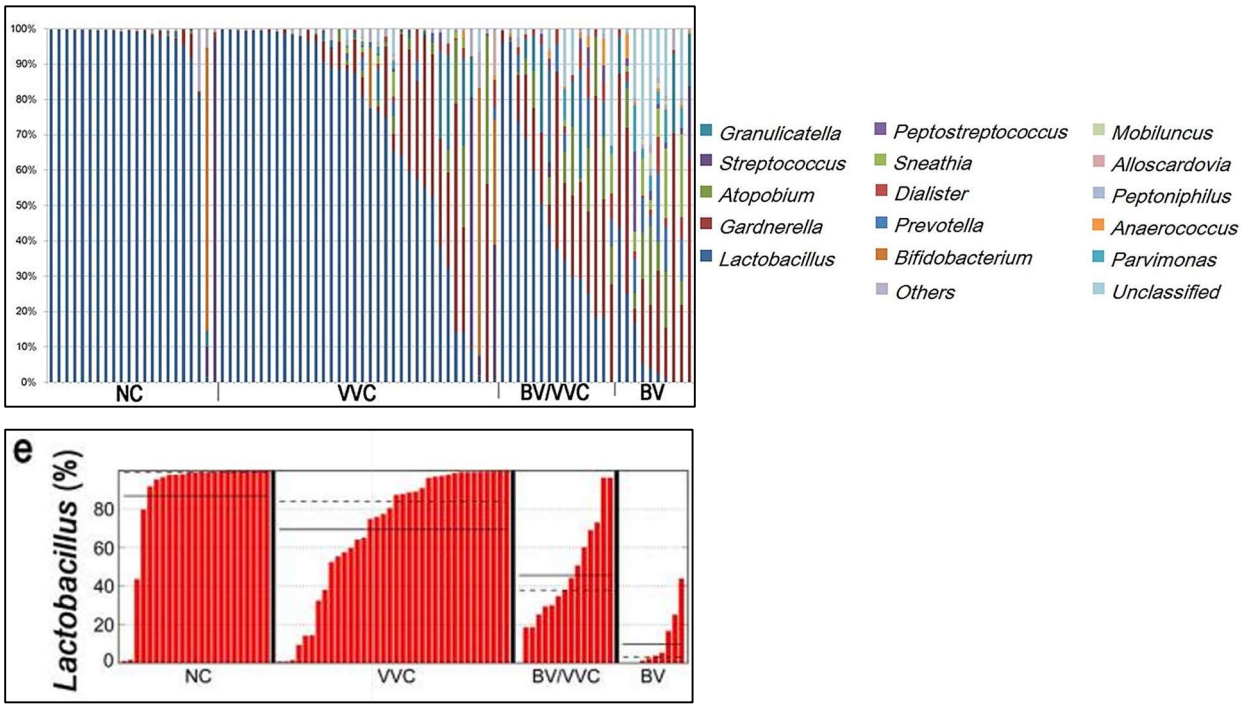

Figure 7 - Genus-level distribution of the vaginal microbiota. Relative abundance of NC, VVC, BV/VVC, and BV. E - Percentage of specific genera (*Lactobacillus*) in NC, VVC, BV/VVC, and BV (Liu *et al.*, 2013).

The precise mechanisms by which vaginal *lactobacilli* inhibit growth of pathogens, including of *Candida* spp. has not been detailed, however, some hypothesis have been raised these being briefly summarized in Figure 8. A more detailed description of these is provided below.

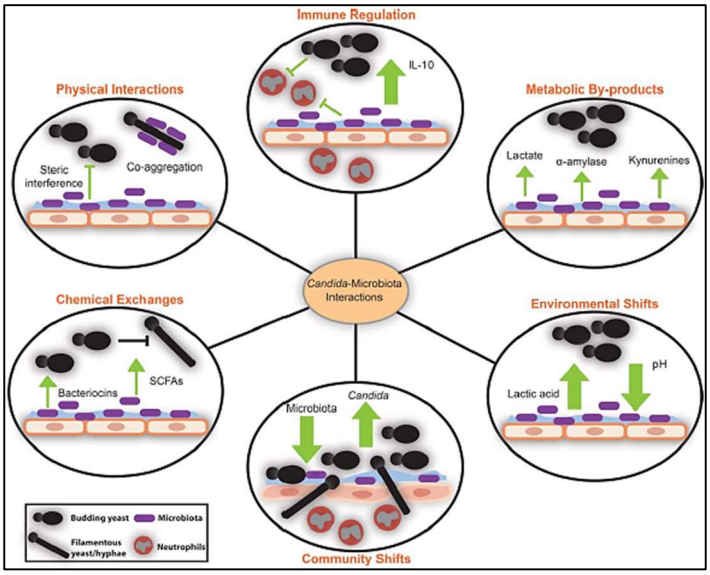

Figure 8 - Interactions between *Candida* and microbiota at the mucosal vaginal surface. Metabolites and small molecules made by the microbiota affect the metabolism and morphology of *Candida* species. Changes in microbiota relative abundance also impact the abundance of *Candida* and its ability to access the mucosal surface, where invasion occurs. In healthy states, when microbiota-derived lactic acid is produced, *Candida* can alter host cytokine production and promote anti-inflammatory signaling (Bradford and Ravel, 2017).

The production of small metabolites that could work as inhibitory molecules for the *Candida spp.* is one of the hypotheses that has been proposed to mediate the *lactobacilli-Candida* interference. The most relevant of these metabolites is lactic acid which is largely produced by lactobacilli during the course of their metabolic activity. In the acidic vaginal niche (pH 4,0-4,5) weak acids, such as the lactic acid are in undissociated form and therefore can enter by passive diffusion into the cells, inducing toxicity and acting as an antimicrobial agent. This happens especially in bacteria because they do not tolerate acidic environments, however in yeasts the same might not be observed since there are generally more tolerant to acidic pHs than bacteria (Jang *et al.*, 2019; Lourenço *et al.*, 2019). However, this simple model by which the production of lactic acid assures by itself protection is being disputed by observations in vitro that show no significant inhibition of *Candida* species by concentrations of lactic acid identical to those observed in vivo (Lourenço *et al.*, 2019) (Figure 9). Furthermore, no correlation between the amount of lactic acid present in the supernatant of lactobacilli cultures and its inhibitory effect against growth of *Candida* was observed (Wang *et al.*, 2017; Lourenço *et al.*, 2019), sustaining the idea that other molecules could be behind the interference effect observed. Acetic acid is also a product of the metabolic activity of *Lactobacillus* however, under eubiosis (that is, in enriched lactobacilli populations), the amount of acetic acid present is low (~4 mM).

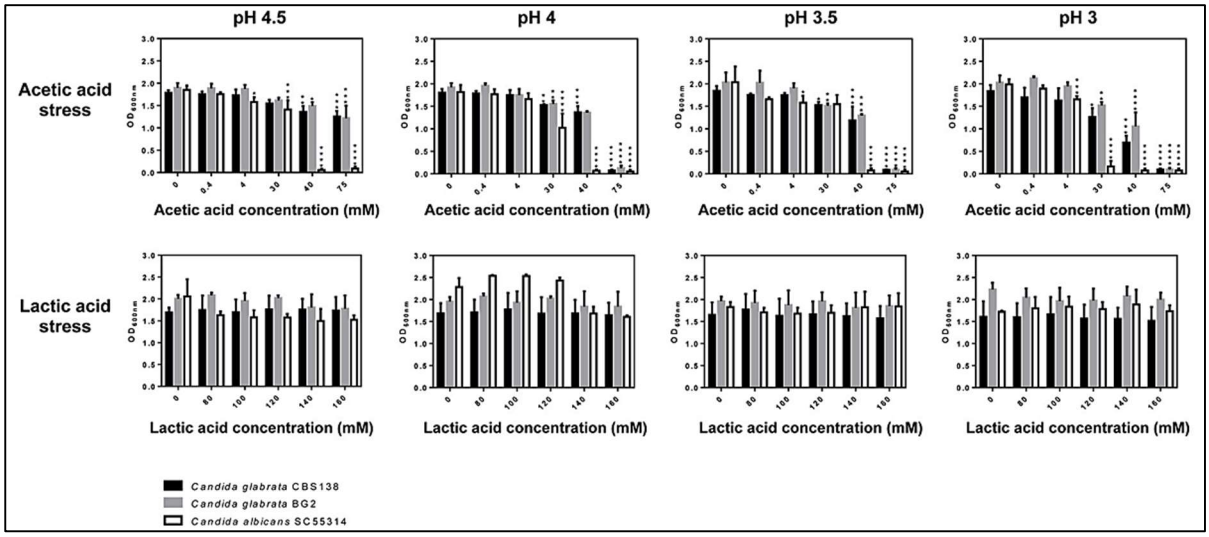

Figure 9 - Growth under microaerophilic conditions of *C. albicans* (white bars), *C. glabrata* CBS138 (black bars) and *C. glabrata* BG2 (gray bars) and with the indicated concentrations of lactic or acetic acid at the pHs depicted in the figure (Lourenço *et al.*, 2019).

Besides organic acids, other compounds produced by *Lactobacilli* are also believed to have some inhibitory effect over *Candida* including H_2O_2 and small peptides known as bacteriocins. As for H_2O_2, the physiological concentration of H_2O_2 present in the vaginal environment is very low and insufficient to exert an inhibitory effect against BV-associated bacteria (O'Hanlon, Moench and Cone, 2011; Gong *et al.*, 2014; Tachedjian *et al.*, 2017) It has also been proposed that the H_2O_2 produced by *Lactobacillus* may contribute to stimulate epithelial cells for the secretion of antimicrobial peptide, exerting by this mechanism its antimicrobial effect (O'Hanlon, Moench and Cone, 2011; Gong *et al.*, 2014; Tachedjian *et al.*, 2017).

The production of exopolysaccharides by *Lactobacillus* has been described to, at least partly, explain their antimicrobial effect against *Candida* species *Candida albicans*, affecting not only growth but also but also virulence traits like filamentation or adhesion (Badel, Bernardi and Michaud, 2011; Allonsius *et al.*, 2017). This is exemplified in results of Figure 10 where it is shown the ability of purified EPS produced by *L. rhamnosus* to inhibit the above-mentioned phenotypic traits of *C. albicans* (Tallon, Bressollier and Urdaci, 2003; Allonsius *et al.*, 2017). This interference of *lactobacilli* over the ability of *Candida* cells to adhere to the surface of epithelial cells may be due to steric hindrance, by blockage of the receptors that could be recognized by *Candida* (but towards which *lactobacilli* may have higher affinity), or by the secretion of molecules that might physically decrease the affinity of fungal proteins involved in adhesion for their corresponding receptors sites (Boris *et al.*, 1998; Boris and Barbés, 2000; Parolin *et al.*, 2015).

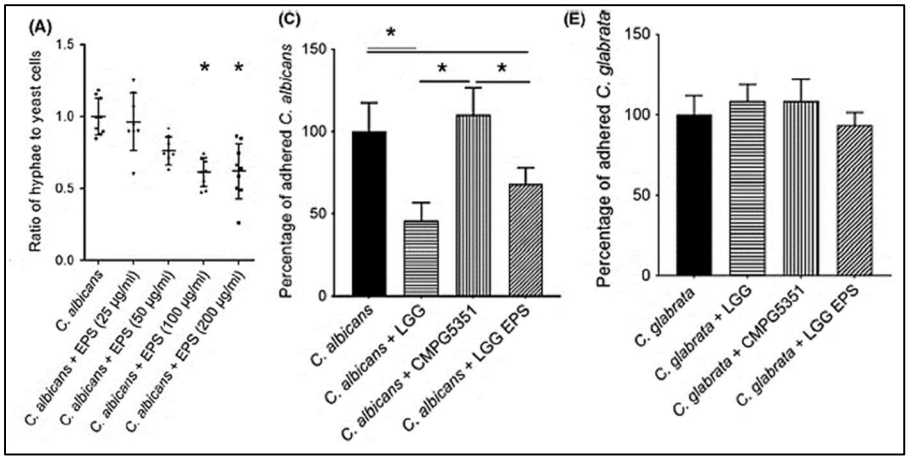

Figure 10 - (A) *Hyphal induction of C. albicans during co-incubation with isolated L. rhamnosus GG EPS. (C, E) Inhibition of Candida adherence to epithelial cells. Percentage of C. albicans and C. glabrata adhered when added in competition with L. rhamnosus GG WT, EPS mutant or L. rhamnosus GG EPS* (Allonsius *et al.*, 2017).

Bacteriocins e biosurfactants

The production of bacteriocins (antimicrobial peptides produced by bacteria) is one of the hallmarks of the physiology of *lactobacilli* and it is being largely explored in the food industries and widely used as an effective food preserving agent (Cleusix *et al.*, 2007; Satpute *et al.*, 2016). Reuterin, produced by *L. reuteri*, and gassericin T, produced by *L. gasseri*, are examples of bacteriocins produced by lactobacilli species that are also a part of the commensal microbiota and that could, at least to some extent, modulate the interaction between these cells. Although the efficacy of reuterin or gassericin T against *Candida* species has not been studied, these small peptides have shown strong activity against several foodborne pathogens (Pandey and Kaushik, 2013).

The secretion of biosurfactants is another mechanism hypothesized to mediate the interference effect of *lactobacilli* over other microbes (Satpute *et al.*, 2016). Biosurfactants are compounds released by microorganisms that facilitate the uptake of water-immiscible substrates and they can also exhibit activity against various microbes including fungi, bacteria and viruses (Boris and Barbés, 2000). Biosurfactants can also be involved in microbial adhesion, acting as anti-adhesive agents (to prevent the adhesion of pathogens to the epithelium). For example, it was observed that in the presence of a biosurfactant produced by *Lactobacillus gasseri* the biofilms produce by several species suffered reduction (Figure 11) (Morais *et al.*, 2017). In particular, it was reported that *L. gasseri* produced biosurfactants with considerable antimicrobial activities against *C. albicans* (Morais *et al.*, 2017). In addition, the amount of a biosurfactant that is required to exert a major effect in the microenvironment is extremely small. Therefore, the role of these compounds in adhesion phenomena may have been previously underestimated.

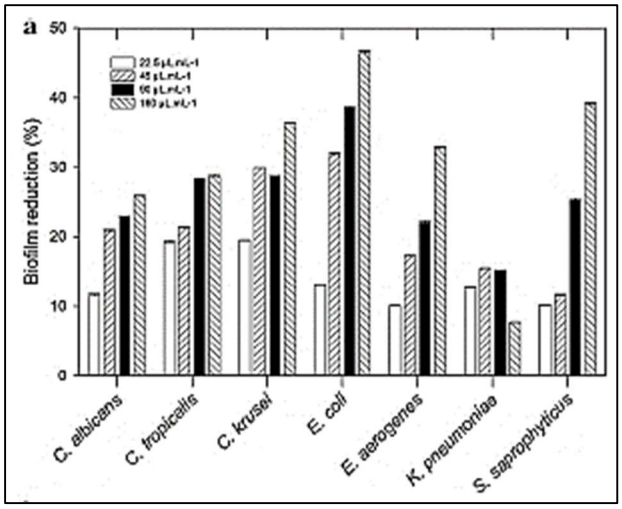

Figure 11 - Percentage of disruption of biofilms produced by pathogenic microorganisms on the surface of polystyrene plates in the presence of different concentrations of biosurfactants produced by *L. gasseriP65* (Morais *et al.*, 2017).

Probiotic formulations already suggested for the treatment of vaginal candidiasis

Due to the long track-record association of *lactobacilli* as a hallmark of vaginal health, various probiotic products have been developed for treatment of vaginal infections. Several clinical trials reported the benefit of some of these commercially available probiotic formulations for women's urogenital health (Mu, Tavella and Luo, 2018). Nonetheless, as shown below many of these probiotics have been developed using species other than those that are indigenous to the vaginal tract.

Table 1 - The already tested species of Lactobacilli and their effectiveness on diverse *Candida* species. Adapted from: (Strus *et al.*, 2005; Falagas, Betsi and Athanasiou, 2006; Martinez *et al.*, 2009; Gil *et al.*, 2010; Khler, Assefa and Reid, 2012; Parolin *et al.*, 2015; Jørgensen *et al.*, 2017; Rossoni *et al.*, 2018)

	C. albicans	***C. glabrata***	***C. krusei***	***C. lusitaniae***	***C. parapsillosis***	***C. tropicalis***
***L. acidophilus*LA-02**	-	-	-	-		-
***L. fermentum* LS15**	-	-	-			-/+
***L. gasseri* LG21**	+	-/+	-/+	+	-	-/+
***L. paracasei*MC 502**	-	-				
***L. reuteri* RC-14**	-	+	-		-	-
***L. rhamnosus* GR-1**	-	-				-/+
***L. salivarius* LS01**	-	-	-			-

(+) has no effect; (-) has effect (inhibition); (-/+) divergence of study's conclusions; spaces in blank are due to lack of information. The evaluation in based on the capability of the *Lactobacillus* tested to inhibit *Candida* at any level.

A limitation to apply probiotics in medicine isn't their success to treat bacterial infection, but yes, the use of allochthonous strains. The ecological origin of the probiotic strain, the performance, and evolutional history should be taken in consideration when choosing a probiotic strain (Walter, 2008). As the colonization isn't required for the health benefits of the strain, the focus to investigate indigenous strains is minor. All of it, including the lack of knowledge of endogenous species and their biology are together a barrier to use them as probiotics. These studies have shown that the use of probiotics is safe, effective and mainly does not lead to resistances as the current treatments cause. Similarity to the inhibitory effect of *Lactobacillus* cells, the supernatant is also believed to have so much or more potential than cells in relation to the control of the proliferation of *Candida*.

1.3. Brief overview on the positioning of *lactobacilli* and *Candida* spp. in the gastro-intestinal micro- and mycobiomes

The gastro-intestinal tract represents an environment that poses some challenges to the colonization by microbial cells including oxygen-limited concentrations, presence of bile salts, low pH conditions caused by the production of gastric acids and the attachment to the epithelium (Rigottier-Gois, 2013; Mu, Tavella and Luo, 2018). It is known for a long time that *lactobacilli* are colonizers of the human gastrointestinal tract, the metagenomic analyses showing that these are dominant in the stomach, in the duodenum or in the jejunum (Vélez, De Keersmaecker and Vanderleyden, 2007; Walter, 2008). Example of species that colonize the gastro-intestinal are *L. acidophilus, L. brevis, L. casei, L. crispatus, L. delbrueckii, L. fermentum*, *L. gasseri, L. johnsonii, L. paracasei, L. plantarum, L. reuteri, L. ruminis species L. rhamnosus and L. salivarius* (Walter, 2008) .

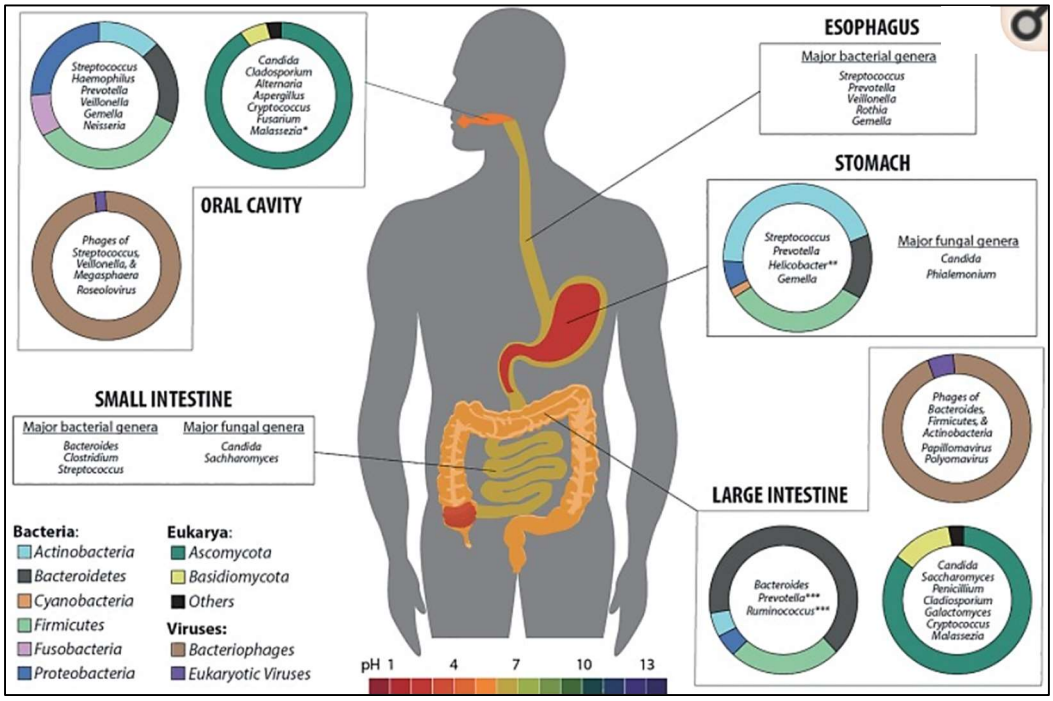

Figure 12 - *Microbiome composition of Bacteria, Eukarya and Viruses among the physiological niches of the human gastrointestinal (GI) tract* (Hillman *et al.*, 2017).

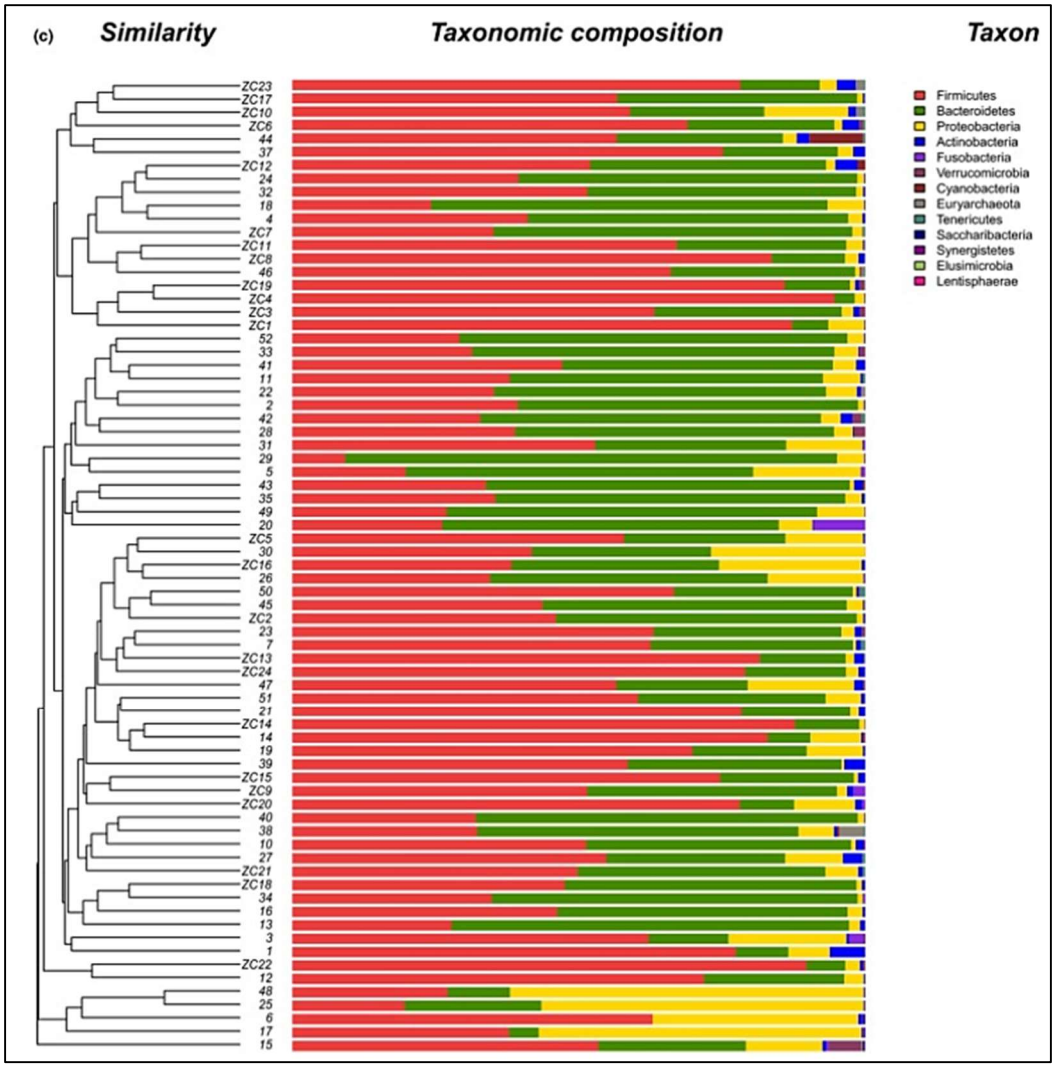

Figure 13 - Relative abundance of gut microbiota at the phylum level in fecal samples (Lun *et al.*, 2019).

However, the identification of indigenous colonizers of the gastro-intestinal tract (usually referred as autochthonous species) is difficulted by the fact that the microbial load observed is also influenced by the species present in ingested foods or beverages (Berg, 2004; Hillman *et al.*, 2017). Indeed, it is thought that most of the *Lactobacillus* species found in the mammalian intestinal tract are not autochthonous but their presence results from the large application of these species by the food industry. The *Lactobacillus* species that are considered indigenous inhabitants of the mammalian intestinal tract include *L. gasseri, L. crispatus, L. reuteri, L. salivarius,* and *L. ruminis* (Reuter, 2001; Walter, 2008), while *L. acidophilus, L. casei, L. paracasei, L. rhamnosus, L. delbrueckii, L. brevis, L. johnsonii, L. plantarum, and L. fermentum* are likely to be allochthonous due to their inability to form stable populations in the gut.

In the gastro-intestinal tract, shotgun sequencing show that fungi make up approximately 0,1% of the total microorganisms (Underhill and Iliev, 2014). The most commonly detected fungi in the gastro-intestinal tract of humans are *C. albicans, Saccharomyces cerevisiae, C. tropicalis, C. parapsilosis, C. glabrata, C. krusei, Cladosporium cladosporioides*, etc. (Lopez-Medina and Y. Koh, 2016). Similar to the *lactobacilli* in the gastro-intestinal tract, a part of the fungal community can be allochthonous, passing through from environmental or dietary exposure without colonizing the gut or exerting any influence on the gut microbiota and host (Suhr and Hallen-Adams, 2015). Among all the existing *Candida* species, *Candida albicans* is the predominant fungal species. Although little interest has been put on these commensal gut *Candida* populations, the more recent studies show that these populations can actually be responsible for triggering disseminated infections (Lopez-Medina and Y. Koh, 2016; Tong and Tang, 2017; Pappas *et al.*, 2018; Wheeler *et al.*, 2019).

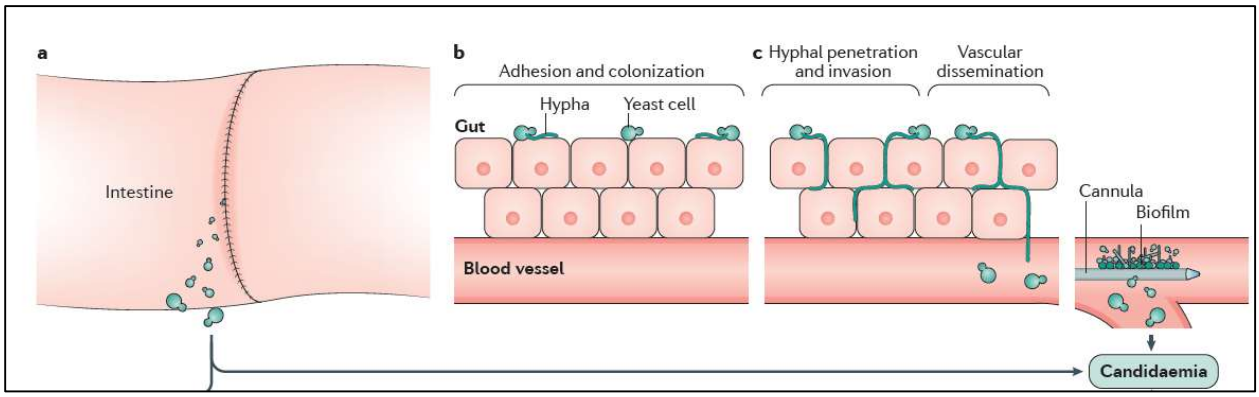

Figure 14 - Pathogenesis of invasive candidiasis. *Candida* spp. can be detected on the mucosal surfaces of ~50–70% of healthy humans. a | When breaches in the intestinal barriers occur, for example, *Candida* spp. can disseminate to the abdominal cavity directly and invade the bloodstream (candidemia). b | Under normal conditions, the fungus behaves as a commensal organism without causing disease. c | Impairment of immune response, among other factors, can promote fungal overgrowth in the gut and candidemia, which can lead to deep-seated opportunistic infections in various organs (invasive candidiasis) (Pappas *et al.*, 2018)

C. albicans penetrates the intestinal epithelium barrier by both fungal-induced endocytosis and active penetration, after which dissemination in the bloodstream occurs (Figure 14) (Koh, 2013; Tong and Tang, 2017). The most important factors increasing dissemination of *C. albicans* from the gut are the lack of an effective host immune response, but an intestinal flora disorder has also been pinpointed as relevant (Prieto *et al.*, 2016; Tong and Tang, 2017; Pappas *et al.*, 2018; Witchley *et al.*, 2019).

Interaction between *Lactobacillus* and *Candida* in the gut

Not much is known about the interference mechanism behind *Candida* and *lactobacilli* in the gut (Strus *et al.*, 2005; Yamaguchi *et al.*, 2005; Gil *et al.*, 2010; Parolin *et al.*, 2015; Förster *et al.*, 2016; Lopez-Medina and Y. Koh, 2016). In the gut, the pH is not as low as the one described for the genito-urinary tract, so here the generic mechanism of inhibition prompted by the accumulation of lactic acid in the undissociated form, is not likely to drive the inhibition. Nonetheless, the possibility that the accumulation of lactate can promote the synthesis of antimicrobial peptides with anti-*Candida* activity by intestinal cells has been proposed (Lopez-Medina and Y. Koh, 2016; Sam *et al.*, 2017). The modulation of the immune system by lactate (immunomodulator effect) may also play a role in the protection of the gut (Hickey *et al.*, 2012; Matsubara *et al.*, 2016). Depletion of adhesion sites or competition for nutrients are other mechanisms hypothesized to underlie the *Candida-lactobacilli* interference in the intestinal mucosa (Vélez, De Keersmaecker and Vanderleyden, 2007; Mason *et al.*, 2012; Förster *et al.*, 2016; Haak and Wiersinga, 2017; Mu, Tavella and Luo, 2018).

1.4. Introduction to the theme of the thesis

Several studies have already demonstrated the protective role of several species of *Lactobacillus* against *Candida* in gastro-intestinal and genito-urinary tract, however, there is a lack of knowledge about the explanatory mechanisms behind. The aim of this thesis, therefore, is to evaluate the effect of two commensal *Lactobacillus* species from the genito-urinary and gastro-intestinal tract, *L. gasseri* and *L. reuteri*, respectively. Starting from previously obtained results on the transcriptomic analysis of a co-culture between *L. gasserii* and *C. glabrata* that led to the identification of 54 *C. glabrata* genes as being over-expressed in the co-culture, compared to their transcript levels in single culture (Ma *et al.*, 2014), in this work we have examined whether three of these genes *CgCTA1*, CAGL0E03498g and CAGL0K04279g, would play a role in determining competitiveness of *C. glabrata* in the presence of *L. gasseri*. To complement this work, a thorough examination of the inhibitory effect exerted by supernatants of *L. gasseri* or *L. reuteri* over growth and virulence traits of *C. albicans* and *C. glabrata* was performed aiming to characterize whether this would be attributable to the lactic acid present or if it resulted from accumulation of another yet uncharacterized metabolite. Finally, it was examined the interaction between *L. reuteri*i, an indigenous intestinal species, and *C. glabrata* and *C. albicans*, and the effect exerted by environmental alterations in this interference mechanism.

Chapter 2 - Materials and methods

2.1. Strains and growth media

The strains used in this work are listed in Table 2.

Table 2 - Description of the group of strains used in this study. The auxotrophy of *C. glabrata* ATCC2001 is his1trp2leu3.

Strain	Description	Source
C. glabrata KUE100	Laboratory strain derived from the reference CBS138	(Bernardo *et al.*, 2017)
C. albicans SC314	Laboratory strain	-
C. glabrata ATCC2001	Reference strain	(Ma *et al.*, 2014)
Cg∆CTA11	*CgATCC2001_∆CAGL0K10868g*	(Ma *et al.*, 2014)
Cg∆CAGL0K04279g	*CgATCC2001_∆CAGL0K04279g*	(Ma *et al.*, 2014)
Cg∆CAGL0E03498g	*CgATCC2001_∆CAGL0E03498g*	(Ma *et al.*, 2014)
L. gasseri ATCC 33323	Reference strain	DSMZ
L. reuteri ATCC 23272	Reference strain	DSMZ

The lactobacilli and the *Candida* species were cultivated in MRS medium which contains, per liter, 10 g/L casein peptone; 10 g/L meat extract; 5 g/L yeast extract; 20 g/L glucose; 1 g/L Tween 80; 2 g/L K_2HPO_4; 5 g/L Na-acetate; 2 g/L Ammonium oxalate; 0.20 g/L $MgSO_4$ x $7H_2O$ and 0.05 g/L $MnSO_4$ x H_2O. Further pH adjustment to 6.2-6.5 at 25CºC was performed. In the case of solid MRS medium, this was acquired from Sigma-Aldrich and was prepared according to manufacturer instruction. In specific, 62 g of the powder were dissolved in 1L water. After this, the media described in this section were sterilized by autoclaving at 121°C, 1 atm, for 15 min. The *Candida* species were also cultivated in RPMI (Roswell Park Memorial Institute), MMB (Minimal medium broth) or YPD (Yeast extract – Peptone – Dextrose) media. RPMI (contains, per liter, 10.8g RPMI-1640 synthetic medium (Sigma), 18g glucose (Merck Millipore) and 34,5g of MOPS (3-(N-morpholino) propane sulfonic acid, Sigma). MMB contains, per liter, 1.70 g yeast nitrogen base (YNB) without amino acids and ammonium (Difco Laboratories, Detroit, Mich.), 2.65 g ammonium sulfate (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). The mutants were cultivated in YPD supplemented with 5mg/L histidine. 12,5mg/L tryptophan and 12,5mg/L leucine.

2.2. Single-species cultivation

To assess individual growth of *L. gasseri, L. reuteri, C. albicans* or *C. glabrata* in liquid MRS medium under microaerophilic conditions, a pre-inoculum of each species was prepared by inoculating 2-3 individual colonies in liquid MRS. The suspensions were cultivated for 24h, at 37ºC, and using an orbital agitation of 100 rpm. After this time, the optical density at 600nm (DO_{600nm}) of these pre-cultures was measured and these cells were used to inoculate fresh MRS medium (50mL) aiming to have an initial OD of 0.1. Growth of the different species was accompanied by following the increase in DO_{600nm} of the cultures. To estimate the number of Colony Forming Units the same experimental setup was used with the difference that each time point the cells were plated every two hours during the first 8h. To count the lactobacilli cells, the culture samples were plated in MRS solid plates and left for 48h at 37ºC in a microaerophilic environment by introducing a candle before selling the container that will create a poor-oxygen atmosphere. To count the *Candida* cells the culture samples were plated in solid YPD medium and incubated at 30ºC under aerophilic conditions. To accompany the formation of lactic acid and the consumption of glucose in the single culture supernatants, appropriate sample volumes were taken along the growth curve. 10 µL of these supernatants were separated by HPLC in an Aminex HPX87H column (Biorad) eluted with a solution of 0.005M sulphuric acid at a flow rate of 0.6 mL/min. A RI (refractive index) detector was used for detection of glucose, lactic acid and ethanol. Appropriate calibration curves using standard solutions were made to estimate the concentrations of glucose, ethanol and lactic acid. In some experiments the procedures were the same but changing the medium used to BHI.

2.3. Multi-species cultivation

Co-cultures of *L. gasseri* or *L. reuteri* with *Candida* strains were performed using an experimental setup similar to the one described above for the single species. In specific, a pre-inoculum of each species was left to grow for 24h in MRS (or, in some cases BHI) liquid medium at 37ºC and 100 rpm. After this time, the cells were inoculated in fresh MRS medium (or, in some cases BHI) aiming to obtain an initial OD_{600nm} of 0.1 for *Candida* species and 0.1, 0.2 or 0.4 for the two species of lactobacilli involved in the co-culture system. Growth of the two species was followed based on the number of CFUs and for this the aliquots of the cultures were plated on: i) MRS solid plates supplemented with 96 mg/L fluconazole (which prevents growth of *Candida*) to assess growth of the two lactobacilli; ii) YPD supplemented with 300 mg/L tetracycline (to inhibit growth of the *lactobacilli*) to assess growth of the *Candida* species. The plates used to assess

lactobacilli growth were put in a box and using a candle in order to have the growths under microaerophilic conditions, at 37ºC for 48h, while the plates used to assess growth of *Candida* were put at 30ºC under aerobic conditions. The formation of lactic acid and the consumption of glucose in the co-culture settings used was performed in a similar manner as described above. Co-cultures of *L. gasseri* or *L. reuteri* with *Candida* strains with induced perturbations were performed using an experimental setup similar to the one described above for the multi species, with the exception that after 6 hours of co-cultivation the pH was increased to 6 (using NaOH), or the temperature was changed from 37ºC to 40ºC or dysbiosis was induced (by supplementation of the medium with tetracycline or fluconazole).

2.4. Effect of lactobacilli culture supernatants in growth of *Candida*

The supernatants obtained along cultivation of *L. gasseri* and *L. reuteri* in MRS medium was examined for its potential to inhibit growth of *Candida* in 200 μL-capacity 96-microwell plates For this, mid-exponential phase *C. albicans* and *C. glabrata* cells cultivated in MMB at pH4 were used to inoculate fresh this same medium supplemented with increasing amounts of the bacterial culture supernatants. The cell suspensions were prepared in 100 μL 2x concentrated MMB medium (at pH 4) to which was added the amount of the bacterial culture supernatant (5,15,25,30,40,50,75 and 100 μL) and sterile distilled water up to a volume of 200 μL. The microplates were incubated at 37ºC at an orbital agitation of 100 rpm. The OD_{600}nm was measured after 2h, 4h, 6h 8h, and 24h of cultivation using a SPECTROstar$_{Nano}$ from BMG LABTECH. The resulting growth curves were used to estimate the kinetic parameters. The effect on growth of *C. albicans* and *C. glabrata* of supernatants obtained after 48h of co-cultivation between *L. gasseri* and *C. albicans* (Ca + Lg) or *C. glabrata* (Cg + Lg) was also tested using the same experimental setup described above.

The effect of the bacterial supernatant culture of C. albicans and C. glabrata growth was also tested adjusting for that the pH of the supernatant to 4 or 6, with HCl or NaOH and using the same experimental setup as described above. The same procedure was done for BHI medium.

2.5. Effect of lactic acid in growth of *Candida*

The effect of lactic acid on growth of *C. albicans* and *C. glabrata* was tested using as sources of the acid a mixture of the D and L isomers (DL-lactic acid) or only L-lactic acid. 4g/L or 8g/L of lactic acid, from both sources, were used to supplement the MRS medium. For this, mid-exponential phase *C. albicans* and *C. glabrata* cells cultivated in MRS at pH4 were used to inoculate fresh this same medium supplemented with different concentrations of lactic acid. The microplates were incubated at 37ºC at an

orbital agitation of 100 rpm. The OD_{600}nm was measured after 2h, 4h, 6h 8h, and 24h of cultivation using a SPECTROstar$_{Nano}$ from BMG LABTECH.

2.6. Extraction and purification of exopolysaccharides obtained from *L. reuteri* cultures

To obtain purified fractions enriched in exopolysaccharides released (EPS-r) to the supernatant or the exopolysaccharide bound to cells (designated as EPS-b) from *L. reuterii*, these cells were cultivated in 10 mL of MRS until an OD_{600nm} of 0.6 was reached. The cellular suspensions were afterwards centrifuged at 15 000 g for 15 min at 4C^0. The pellet (EPS-b) was resuspended in 1mL of PBS followed by a centrifugation at 11000g for 15 min. After that, the pellet was washed in 5 ml of sterile sodium chloride solution and then resuspended in 5 ml of 0.05 M EDTA to obtain the bound EPS fraction. The mixture was incubated under gentle agitation for 4 h at 4C^0 and then centrifuged at 6000 g for 30 min at 4 ◦C. The EPS-b was precipitated from the supernatant by addition of two volumes of cold ethanol followed by an overnight incubation at 4Cº. After this time, the suspension was centrifuged during 30 min at 6000 g, at 4 ºC, and the EPS-b obtained was resuspended in 2 ml of distilled water. Afterwards this b-EPS was dialyzed (molecular weight cut-off: 6000–8000 Da) against 5L of distilled water for 2 days with three water changes per day. To obtain the EPS released into the culture broth, the supernatant resulting of the first centrifugation was used (EPS-r). Afterwards, 2mL of 20% trichloroacetic acid was added and the mixture was incubated for 2h at 4Cº under gentle agitation. The precipitated proteins were removed by centrifugation at 25 000 g for 20 min at 4 ºC and the EPS was precipitated from the supernatant using two volumes of cold ethanol followed by an overnight incubation at 4 ºC. After this time, the suspension was centrifuged at 6000 g for 30 min at 4Cº and the pellet containing EPS-r was resuspended in 2 ml of distilled water and dialyzed as described for the EPS-b fraction. The total amount of carbohydrates present in the EPS was determined using the phenol/sulfuric acid method and using glucose as a standard. The quantification of EPS was expressed in g/L, equivalent of glucose per liter of growth medium. The purified EPS-b and EPS-r fractions were used to supplement the MRS growth medium where *C. albicans* and *C. glabrata* were cultivated (0 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml. For this, mid-exponential phase *C. albicans* and *C. glabrata* cells cultivated in MMB at pH4 were used to inoculate fresh this same medium supplemented with increasing amounts of EPS. The cell suspensions were prepared in 100 µL 2x concentrated MMB medium (at pH 4) to which was added the amount of EPS and sterile distilled water up to a volume of 200 µL. The microplates were incubated at 37ºC at an orbital agitation of 100 rpm. The OD_{600}nm was measured after 2h, 4h, 6h 8h, and 24h of cultivation using a SPECTROstar$_{Nano}$ from BMG LABTECH.

2.7. Effect of bacterial supernatant/or of bacterial cells in the ability of *C. albicans* to induce filamentation

The effect of a 48h *L. reuteri* culture supernatant or of the *L. reuteri* live cells on the ability of *C. albicans* cells to trigger filamentation was tested in: a) RPMI medium also inoculated with *L. reuteri* cells (OD=0,4) (before cultivated in MRS medium) in a proportion of 1:4 (yeast:bacteria); b) in RPMI supplemented with 50% bacterial culture supernatant; c) RPMI supplemented with 10% FBS (fetal bovine serum). All the observations were obtained with a Zeiss microscope using a 1000x magnification. After inoculation of the C. albicans cells at an OD_{600nm} of 0.1 under the different conditions, samples of the cultures were taken for microscopic analysis. All the observations were obtained with a Zeiss microscope.

2.8. Effect of bacterial supernatant in *C. glabrata* and *C. albicans* biofilm formation

The effect of the 48h *L. reuteri* culture supernatant in the ability of *C. glabrata* and *C. albicans* cells to form biofilms was performed using an experimental setup similar to the one described in 2.4. Briefly, it was cultivated *L. reuteri* cells in MRS for 48 hours and then the supernatant was harvested. It was cultivated cells of *C. albicans* and *C. glabrata* in MMB and to these were added increasing volumes of the supernatant collected (25, 50, 100µL). The microplates were incubated with an orbital agitation of 25 rpm at 37ºC for 8h and 24h. After these times, the medium was carefully removed from each well and cells were washed twice with 100 µL PBS (Phosphate-buffered saline). All the observations were obtained by SEM (Scanning Electron Microscopy) using a magnification between 200x and 20000x with a high voltage of 20.0 kV.

Chapter 3 – Results

3.1. Identification of competitiveness genes mediating the interaction of *C. glabrata* with *L. gasseri*

A previous transcriptomic analysis that surveyed the alterations in genomic expression of *L. gasseri* and *C. glabrata* in a co-culture setting in MRS medium (after 2h or 8h of cultivation) unveiled a strong up-regulation in the co-culture setting of the *CgCTA1* gene, predicted to encode a catalase, and of the poorly characterized ORFs CAGL0E03498g and CAGL0K04279g. In specific, these genes were found to be up-regulated in the co-culture setting by 1,91, 1,72 or 2,61-fold, in comparison with the transcript levels achieved in single-culture. Based on these observations it was decided to examine whether the expression of these three genes would contribute to increase competitiveness of *C. glabrata* while growing the presence of *L. gasseri*. As such, wild-type cells and three derived strains devoid of *CgCTA1*, CAGL0E03498g or CAGL0K04279g genes were cultivated in MRS medium in single-culture or in co-culture with *L. gasseri*. The results obtained are shown in Figure 15. Minor differences were observed in the growth rate of the three *C. glabrata* strains tested while growing in single-culture, although along time a higher loss of viability, in comparison with the one exhibited by wild-type cells (Figure 15). In the presence of *L. gasseri* the strain devoid of CgCTA1 showed a lower viability than wild-type cells suggesting that the expression of this gene could contribute to improve survival of the yeast in the presence of the bacteria (Figure 15). A mildly reduced growth was also observed for the strains devoid of the CAGL0E03498g and CAGL0K04279 gene (Figure 15), although in this cases this could also result from the observed poorer fitness of this strains even when cultivated alone in MRS (Figure 15). Interestingly, viability of the *L. gasseri* cells in the presence of *C. glabrata* was maintained higher along the co-cultivation. Consistent with the above described phenotypes, this effect was more visible for the wild type and ΔCAGL0K04279g strains (Figure 15).

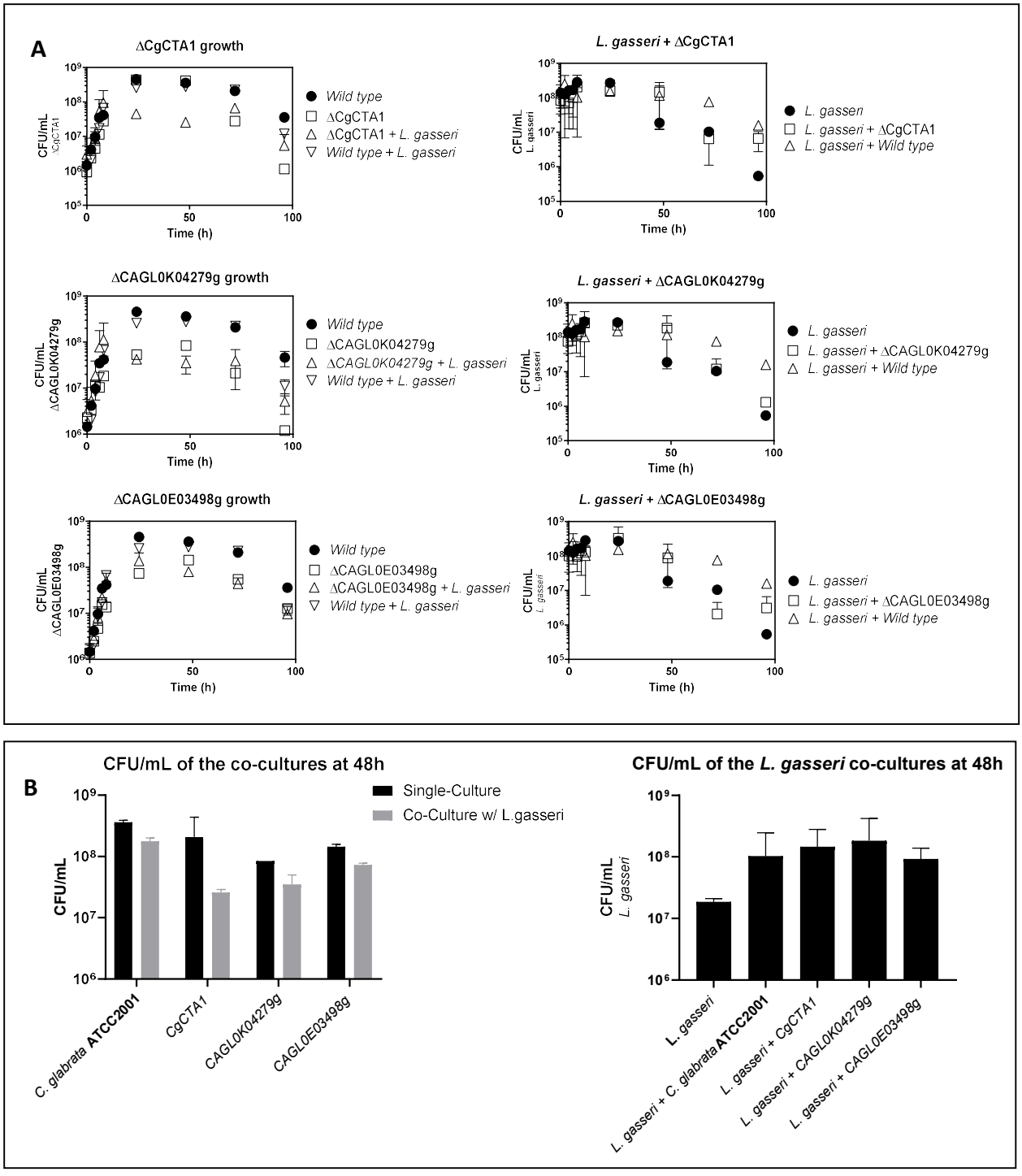

Figure 15 - Growth (CFU/mL) of mutants CgCTA1, CAGL0K04279g and CAGL0E03498g with *L. gasseri*. The control is the wildtype (*C. glabrata* ATCC2001). Panel A - CFU/mL of single cultured and co-cultured mutants with *L. gasseri*. Panel B - Viability in CFU/mL at 48h of mL of single cultured and co-cultured mutants with *L. gasseri*, pH 4, 37ºC at 100 rpm. The *Candida*'s growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The *L. gasseri* growth was determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole. The results shown were obtained from three independent experiments. Without significant statistic values.

3.2. Tacking the inhibitory effect of *L. gasseri* supernatant over growth of *C. albicans* and *C. glabrata*

Previously it has been demonstrated that a supernatant of a *L. gasseri* culture has an inhibitory effect on growth of *C. albicans* and *C. glabrata* (Pedro, 2017), as also demonstrated to occur in the case of other lactobacilli species (Parolin *et al.*, 2015; Tan *et al.*, 2017; Vahedi-shahandashti, Kasra-kermanshahi and Shokouhfard, 2017). In order to get further insights into this, in this thesis it was examined whether this inhibitory effect would be modulated by the pH or by the presence of *Candida* species. As such, we have compared the inhibitory effect prompted by the *L. gasseri* supernatant (obtained after 48h of cultivation in MRS, where the pH was decreasing along time, having a final pH ~4) or in this same supernatant adjusted to pH 7 (using NaOH for tittering the pH) in growth of *C. albicans* and *C. glabrata* in MRS medium. The results obtained showed a dose-dependent effect of both supernatants in inhibition of *C. albicans* and *C. glabrata* growth (Appendix 2 and Appendix 3), a much stronger effect being observed when the pH of the supernatant was maintained in an acidic state (pH ~4) (Figure 16).

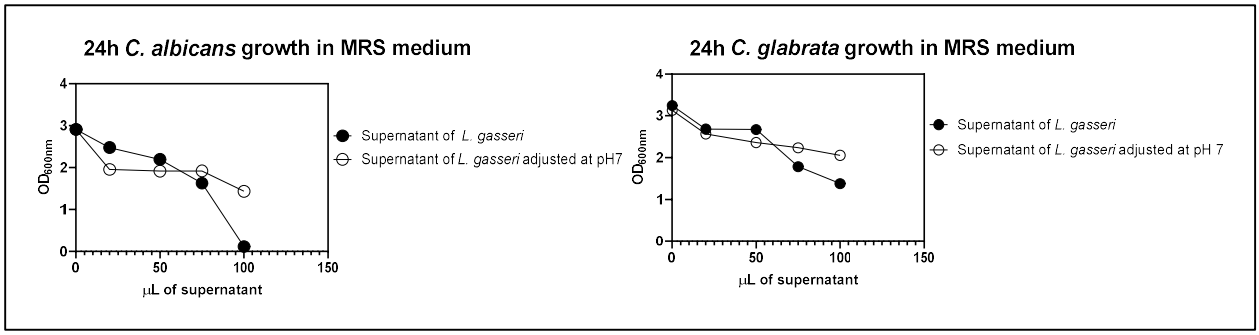

Figure 16 - 24-hour growth curve of *C. albicans* and *C. glabrata* with different concentrations of *L. gasseri* supernatant in MRS medium at pH 4 and 7 (adjusted), at 37ºC with 100 rpm.

In order to assess whether this pH-dependent effect was specific of the MRS medium, we have also assessed the inhibitory effect of a *L. gasseri* culture supernatant obtained after 48h of incubation in BHI medium. The results obtained demonstrated again a dose-dependent inhibitory effect of this supernatant in inhibiting growth of *C. albicans* and *C. glabrata* (Appendix 4 and Appendix 5), however, in this case a higher effect was observed for the supernatant that was maintained at pH 7 (Figure 17).

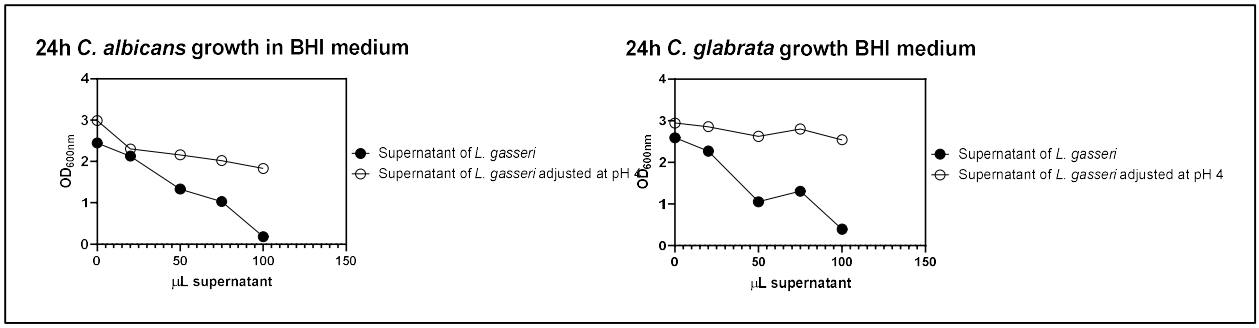

Figure 17 - 24-hour growth curve of *C. albicans* and *C. glabrata* with different concentrations of *L. gasseri* supernatant in BHI medium at pH 4 (adjusted) and 7, at 37ºC with 100 rpm.

Since the inhibitory effect of *lactobacilli* over growth of *Candida* has been largely attributed to the production of lactic acid and considering the above demonstrated effect of pH in modulating the inhibitory effect of the bacterial culture supernatants, we have examined whether the supplementation of the MRS medium with the concentrations of lactic acid usually present in *Lactobacillus* culture supernatants (ranging between 4 and 8 g/L, when 20 g/L glucose are used as carbon source) would lead to the same inhibitory effect as observed with the supernatants. The results obtained clearly demonstrate that is not the case since the supplementation of the MRS medium with the isomers of lactic acid (DL or L) did not resulted in a significant inhibition of growth for the two *Candida* species (Figure 18 and Appendix 6).

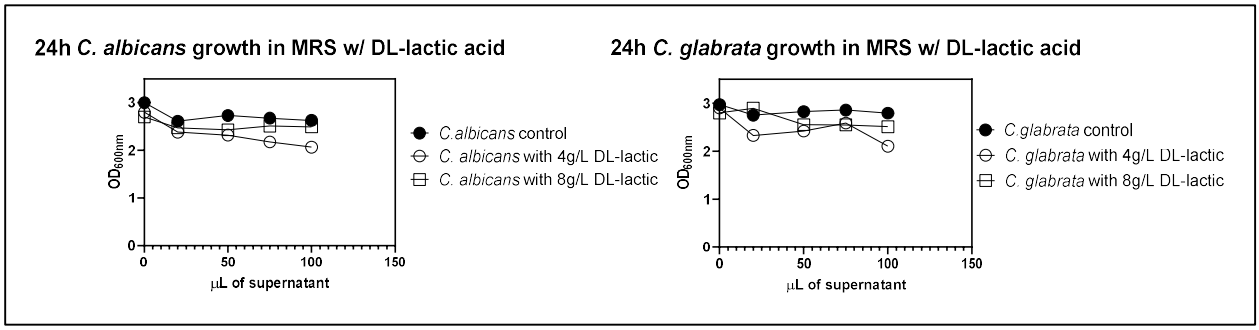

Figure 18 – Growth curve of *C. albicans* and *C. glabrata* in MRS medium with 4g/L and 8g/L of DL-lactic acid, at 37ºC with 100 rpm.

Another aspect that we have also investigated was whether a supernatant obtained from a co-culture between *C. albicans or C. glabrata* and *L. gasseri* would be more inhibitory than the supernatant of a single bacterial culture. The results demonstrated no significant effect with the inhibition obtained with this supernatant obtained from the co-culture exerting a toxic effect that was identical to the one obtained with the supernatant obtained from the single-culture (Figure 19 and Appendix 7).

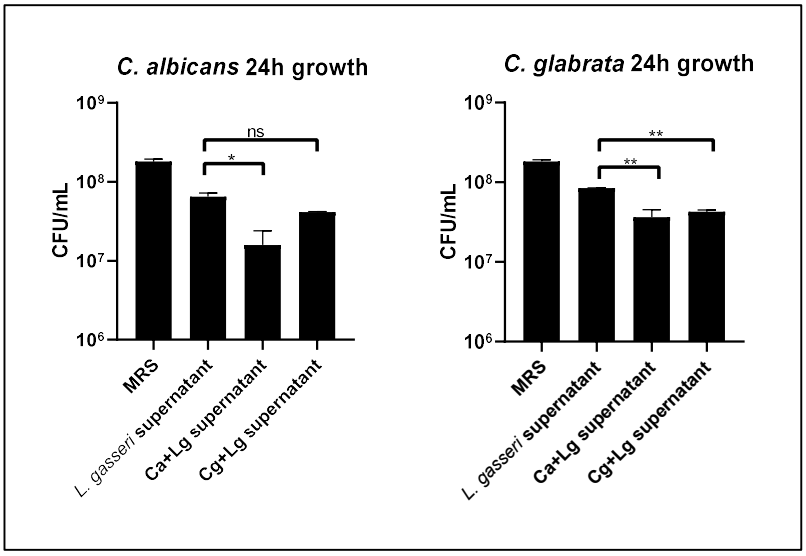

Figure 19 - Viability at 24h in CFU/mL of *C. albicans* and *C. glabrata* with *L. gasseri* supernatant and co-culture supernatant (*C. albicans* with *L. gasseri* – Ca+*Lg*, *C. glabrata* with *L. gasseri* – Cg+*Lg*) in MRS medium at pH 4 at 37ºC with 100 rpm. The control is carried out in MRS medium. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The results shown were obtained from three independent experiments.

Effect of environmental changes in the modulation of the *C. glabrata-L. gasseri* interaction

Because alterations in the environment can significantly contribute to alter the homeostasis between *Candida* species and *L. gasseri*, we have explored the established experimental setup for co-cultivation of *L. gasseri* and *C. albicans/C. glabrata* to investigate the impact of changing: i) increase the pH to 6; ii) increase the temperature from 37ºC to 40ºC; iii) presence of a lactobacilli-killing antibiotic (tetracycline). The results obtained showed that the increase in the pH to 6 did not lead to changes of viability of the yeast species or of *L. gasseri* (Figure 20) while the increase in the temperature had little effect in viability of the two *Candida* species but greatly accelerated loss of viability in *L. gasseri* (Figure 21).

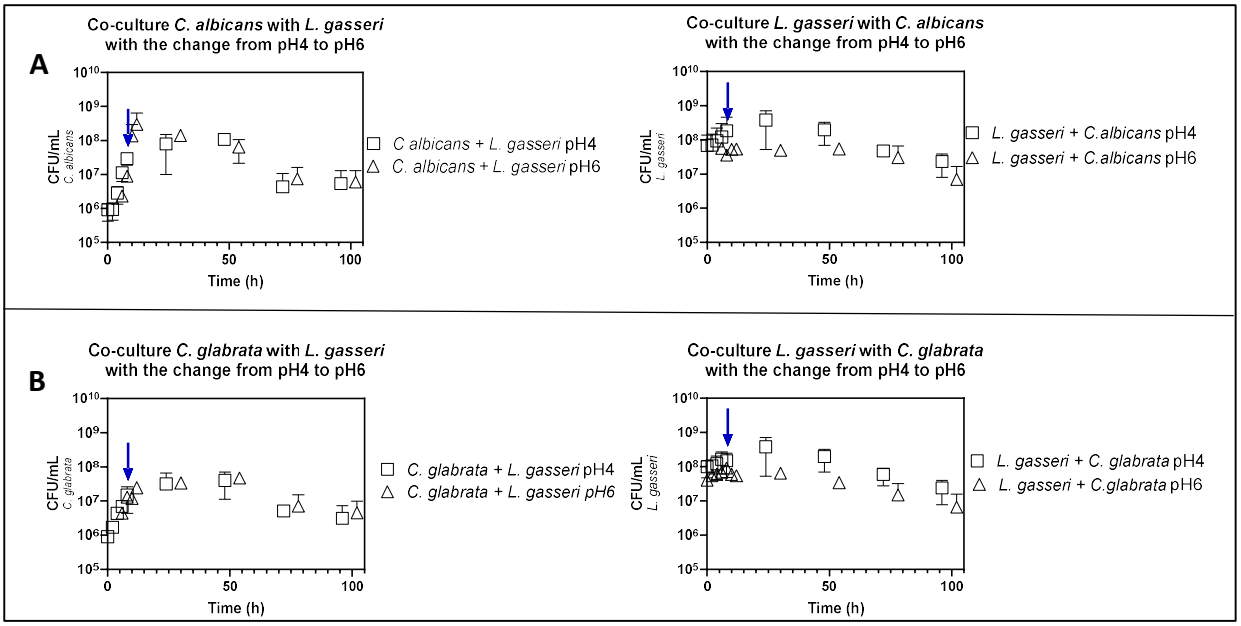

Figure 20 - Growth (CFU/mL) of *C. glabrata* with *L. gasseri* with an alteration of pH from pH 4 to pH 6, after 6h of culture; adjusted with NaOH. Panel A - CFU/mL of *C. albicans* and *L. gasseri* in co-culture. Panel B - CFU/mL of *C. glabrata* and *L. gasseri* in co-culture. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The *L. gasseri* growth was determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole. The arrow indicated the introduction of the perturbance. The results shown were obtained from three independent experiments.

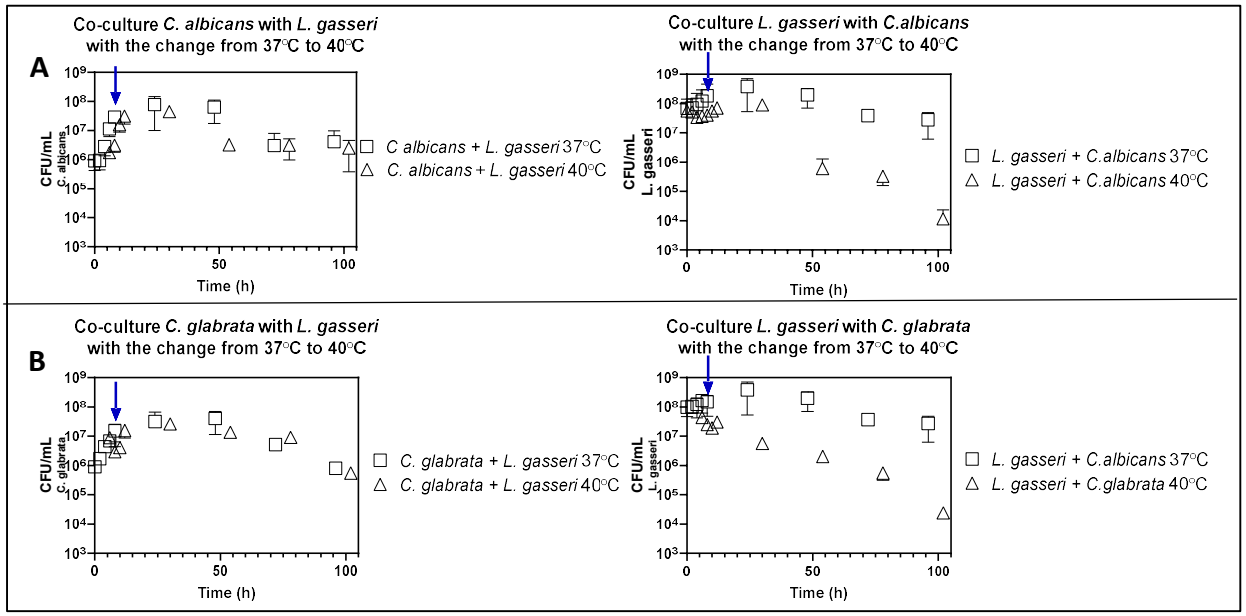

Figure 21 - Growth (CFU/mL) of *C. glabrata* with *L. gasseri* with an alteration of temperature from 37ºC to 40ºC, after 6h of growth. Panel A - CFU/mL of *C. albicans* and *L. gasseri* in co-culture Panel B - CFU/mL of *C. glabrata* and *L. gasseri* in co-culture. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The *L. gasseri* growth was determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole. The arrow indicated the introduction of the perturbance. The results shown were obtained from three independent experiments.

To mimic what can be a dysbiosis condition, 600 mg/L of tetracycline were added to an established co-culture being possible to observe that the concentration of antibiotic used indeed resulted in a decline of the *L. gasseri* population present (Figure 22 and Figure 23). Consistent with the results obtained until so far, the decrease in *L. gasseri* population induced by the presence of the antibiotic correlated with an increase in the viability of the *Candida* species, this being particularly evident for *C. glabrata* (Figure 22).

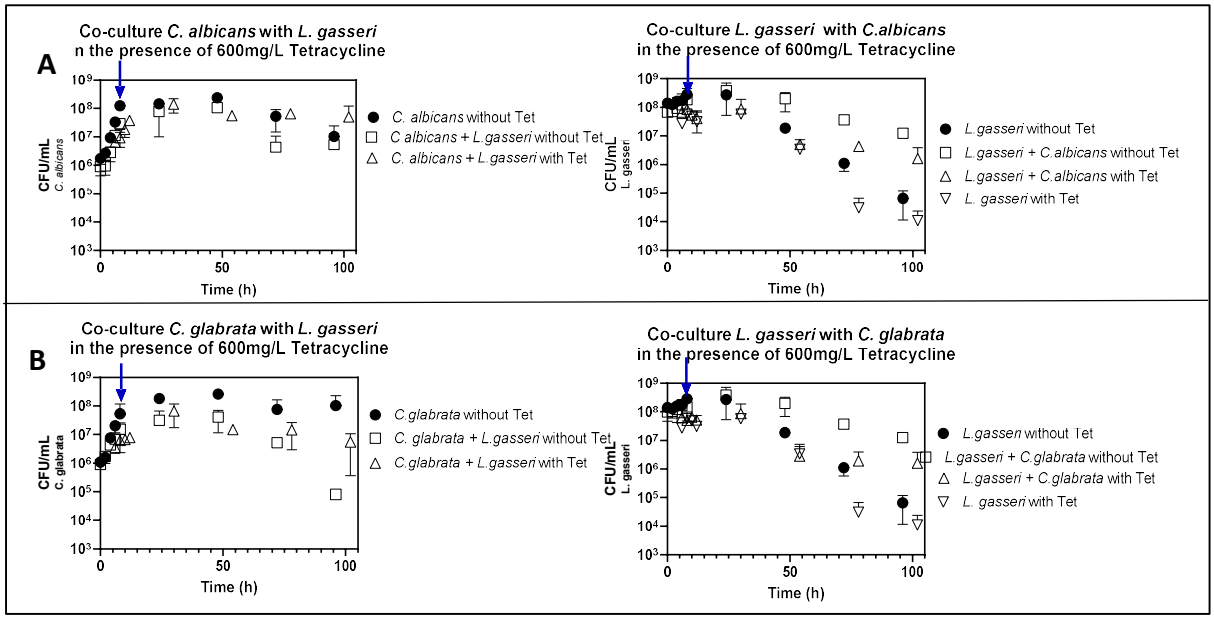

Figure 22 - Growth (CFU/mL) of *C. albicans* and *C. glabrata* with *L. gasseri* with an addiction of 600mg/L of tetracycline, after 6h of culture. Panel A - CFU/mL of *C. albicans* and *L. gasseri* in co-culture. Panel B - CFU/mL of *C. glabrata* and *L. gasseri* in co-culture. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The *L. gasseri* growth was determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole. The arrow indicated the introduction of the perturbance. The results shown were obtained from three independent experiments

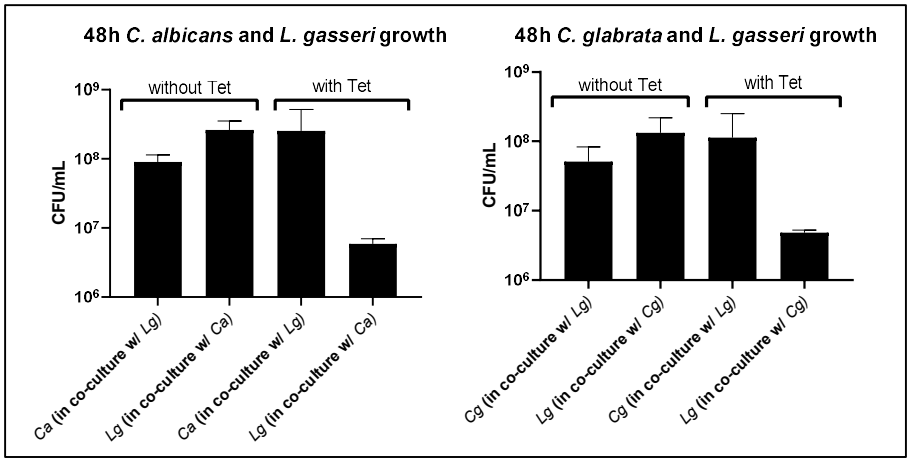

Figure 23 – Viability at 48h in CFU/mL of *C. albicans/C. glabrata* and *L. gasseri* in co-culture at pH 4 at 37ºC without or with tetracycline, with 100 rpm. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The results shown were obtained from three independent experiments. Without significant statistic values.

3.3. Effect of *L. reuteri* on growth of *C. albicans* and *C. glabrata*

The work undertaken in the laboratory until so far was focused on the inhibitory effects exerted by *L. gasseri* over *C. albicans and C. glabrata* but in this thesis this study was extended to the *L. reuteri* species. To assess the effect of *L. reuteri* on growth and physiology of *C. albicans* and *C. glabrata*, co-culture experiments were envisaged using an experimental setup similar to the one that was already used for *L. gasseri.* Growth of *L. reuteri*, *C. albicans* and *C. glabrata* in single and in co-culture was followed in MRS medium during 96h and the result obtained are shown in Figure 24 and Figure 25. The first observation that comes out of these results is the loss of viability that is observed in the *L. reuteri* population either in single and co-culture, this being more pronounced in the single-culture (in line with the also observed "protective" effect exerted by the presence of the two *Candida* species) (Figure 24). This was also accompanied by a reduction in the viability of the two *Candida* species, particularly in the case of *C. albicans* that suffered a very prominent reduction in viability (around 46,7%-fold reduction, compared to the maximum number of viable cells achieved in the co-culture). As also observed in the case of *L. gasseri*, the fact that the reduction in viability is observed for the later periods of cultivation suggests the accumulation of one or more inhibitory products in the supernatant that, in the end, contribute to reduce the viability of the *Candida* species.

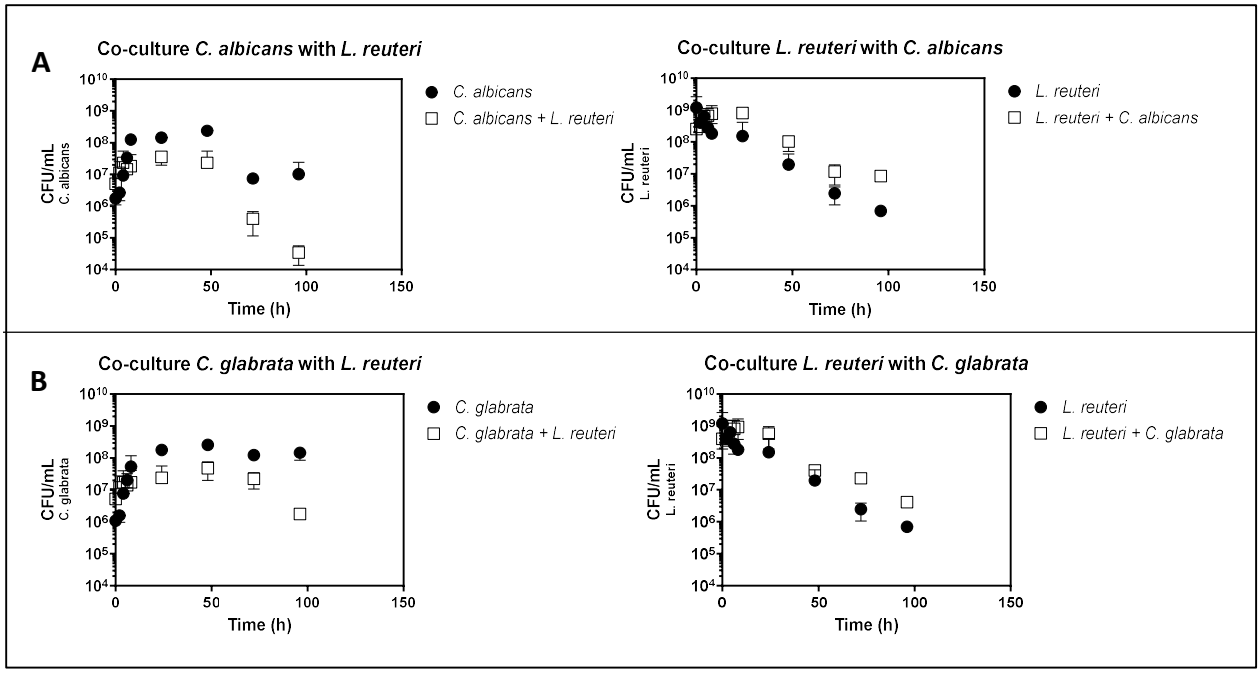

Figure 24 - Growth (CFU/mL) of *C. albicans* and *C. glabrata* with *L. reuteri*. Panel A - CFU/mL of *C. albicans* and *L. reuteri* in co-culture. Panel B) - CFU/mL of *C. glabrata* and *L. reuteri* in co-culture. The *L. reuteri* growth was determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The results shown were obtained from three independent experiments.

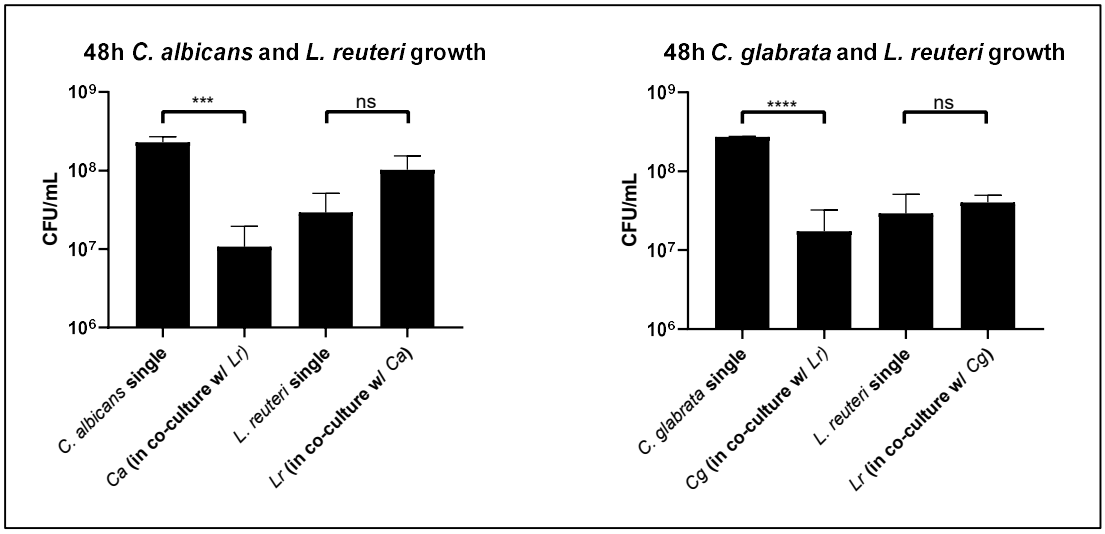

Figure 25 - Viability in CFU/mL at 48h of *C. albicans* and *C. glabrata* and *L. reuteri* in co-culture, pH 4, 37ºC with 100 rpm. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The results shown were obtained from three independent experiments.

Based on the above described observations that show reduced viability of *C. albicans and C. glabrata* in the later stages of the co-culture and also on the above shown inhibitory effect over growth of these two yeasts of a *L. gasseri* culture supernatant, we have decided to examine whether a similar inhibitory effect could be observed using a *L. reuteri* supernatant. As such, we have started by examining the effect of a supernatant obtained after 48h of cultivation of *L. reuteri* in MRS medium on growth of *C. albicans* and *C. glabrata* in MRS medium. The results obtained, shown in Figure 26 show a significant inhibitory effect exerted by the presence of the bacterial supernatant, especially in the case of *C. albicans* whose growth was more significantly inhibited (Figure 26 - A).

Having shown that there is an inhibitory potential for *L. reuteri* supernatants obtained from cultivation of the bacteria in MRS medium, we have examined whether this inhibitory effect was cumulative along time. The results obtained, shown in Figure 26 - B, confirmed that this is the case with the supernatants obtained at later stages of *L. reuteri* cultivation being much more inhibitory than those obtained at earlier stages. Like it was also observed for *L. gasseri*, the inhibitory effect of the *L. reuteri* supernatant obtained after cultivation in MRS medium, was significantly reduced when the pH of this was increased to 7 (Figure 26 - C, Appendix 8 and Appendix 9). Under the conditions we have used to cultivate *L. reuteri* the maximum amount of lactic acid present in the supernatants was of 8 g/L which, as shown in Figure 18, has no significant inhibitory effect over growth of *C. albicans* or *C. glabrata* (Lourenço *et al.*, 2019).

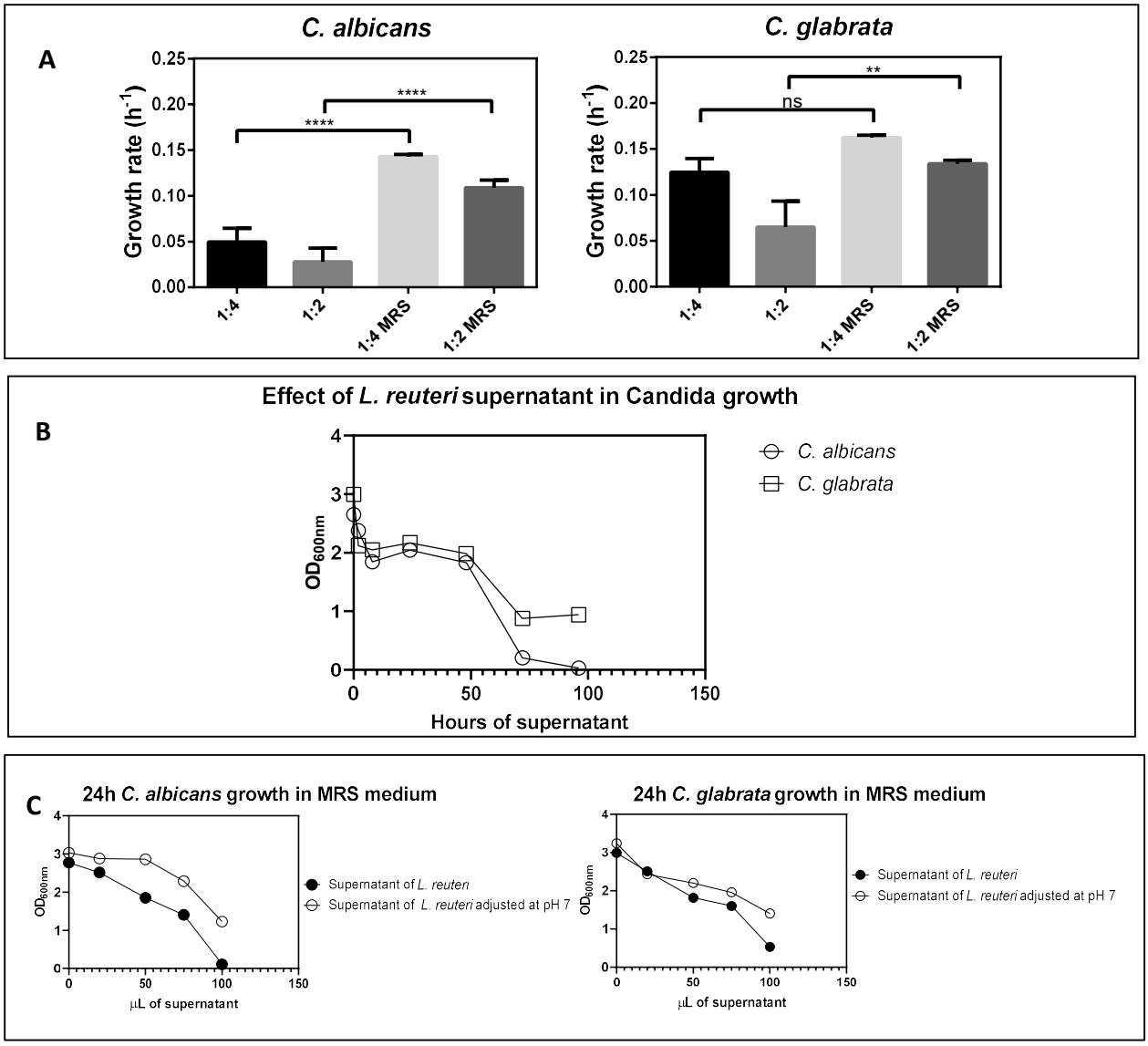

Figure 26 – Panel A - Growth rate of *C. albicans* (right) and *C. glabrata* (left) in the presence of 1:4 or 1:2 *L. reuteri* supernatant and 1:4 or 1:2 MRS medium (control). Panel B - Growth curve of *C. albicans* and *C. glabrata* with *L. reuteri* supernatant harvested in different times (0h, 2h, 4h, 8h, 48h, 72h, 96h) in MRS medium at pH 4 at 37ºC with 100 rpm. Panel C - 24-hour growth curve of *C. albicans* and *C. glabrata* with different concentrations of *L. reuteri* supernatant in MRS medium at pH 4 and 7 (adjusted), at 37ºC with 100 rpm. The results shown were obtained from three independent experiments. Where each result is derived from an average value.

Interestingly, the inhibitory effect of a supernatant obtained from a co-culture between *L. reuteri and C. albicans* was more inhibitory than a supernatant obtained from a *L. reuteri* single-culture (Figure 27 – A and B) and this effect was also augmented as the supernatant was obtained from later stages of the co-cultivation (Figure 27 - C).

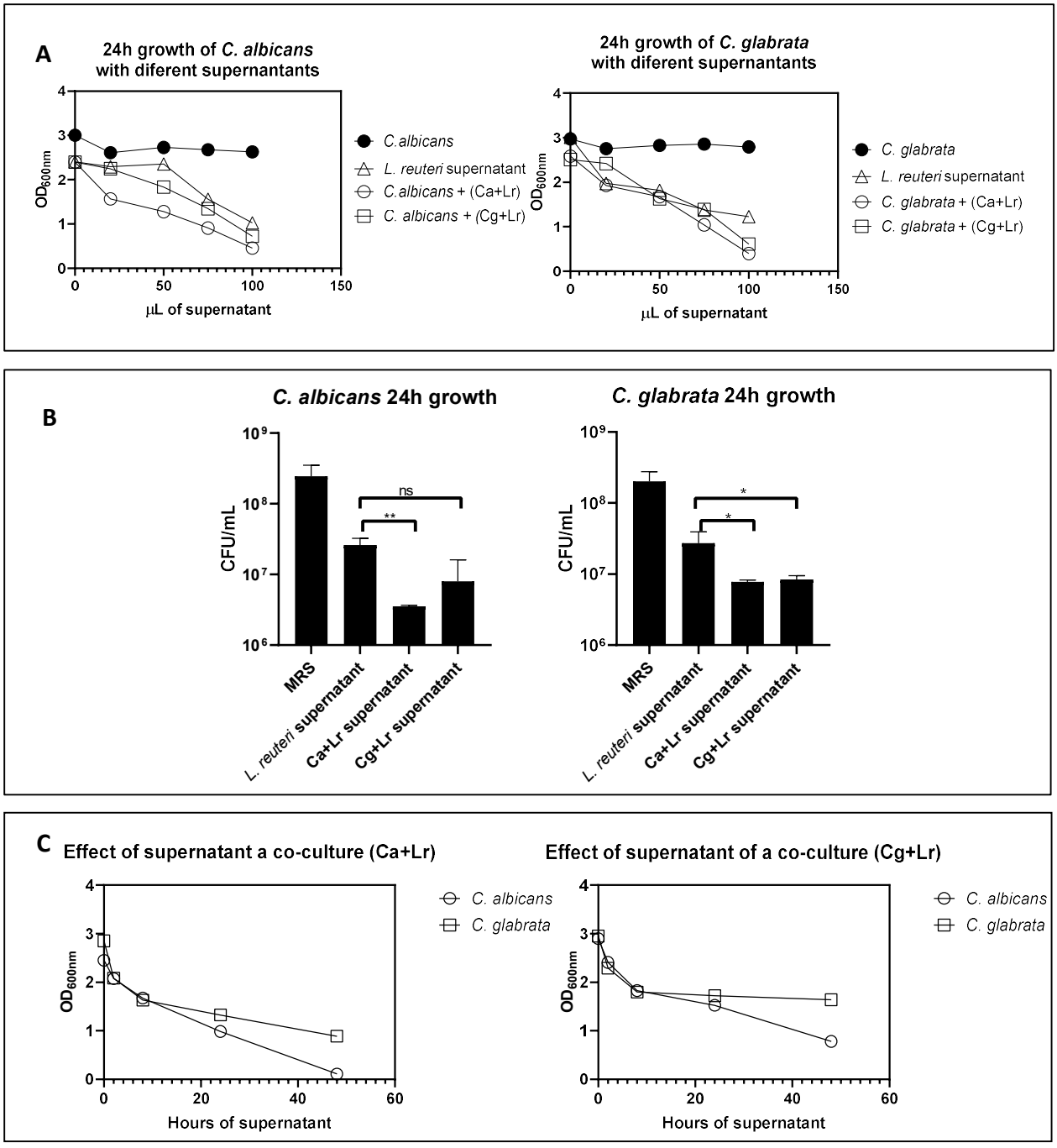

Figure 27 – Panel A - Growth curve and Viability in CFU/mL (Panel B) of *C. albicans* and *C. glabrata* with different concentrations of a *L. reuteri* supernatant and co-culture supernatant (*C. albicans* with *L. reuteri* – Ca+*Lr*, *C. glabrata* with *L. reuteri* – Cg+*Lr*) in MRS medium at pH 4 at 37ºC with 100 rpm. The control is carried out in MRS medium. The *L. reuteri* growth was determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. Panel C - Growth curve of *C. albicans* and *C. glabrata* with supernatant of a co-culture harvested in different times (0h, 2h, 8h, 24h, 48h) in MRS medium at pH 4 at 37ºC with 100 rpm. The results shown were obtained from three independent experiments. Where each result is derived from an average value.

Effect of *L. reuteri* culture supernatant or of *L. reuteri* live cells in virulence traits of *Candida*

Our results (Pedro, 2017) and others (Tropcheva *et al.*, 2014; Matsubara *et al.*, 2016; Tan *et al.*, 2018) have demonstrated a significant inhibitory effect of lactobacilli culture supernatants in inhibiting filamentation undertaken by *C. albicans*, this being a highly relevant virulence trait of this species. As such in this work we have examined whether supernatants obtained from *L. reuteri* cultures or even *L. reuteri* cells exhibited a similar capacity to modulate filamentation undertaken by *C. albicans*. For this, the yeast cells were cultivated in RPMI pH 7 supplemented with increasing concentrations of the *L. reuteri* culture supernatant or with *L. reuteri* live cells (10^8 CFUs/mL). The experiments were also performed supplementing the RPMI medium with 10% FBS since this is a known potent inducer of filamentation in *C. albicans* (Azadmanesh *et al.*, 2017). The cells were counted under microscope analysis and the results obtained clearly demonstrated a clearly reduced ability of the *C. albicans* cells to undergo filamentation when cultivated in the presence of the *L. reuteri* supernatant, this being observed either in the presence or absence of FBS (Azadmanesh *et al.*, 2017). In the presence of *L. reuteri* live cells the filamentation was also reduced, compared to the control, this phenotype being clearly alleviated when the *L. reuteri* cells are killed before inoculation in the RPMI medium (Figure 28). Representative images obtained in the microscopy analysis undertaken are shown in Figure 29.

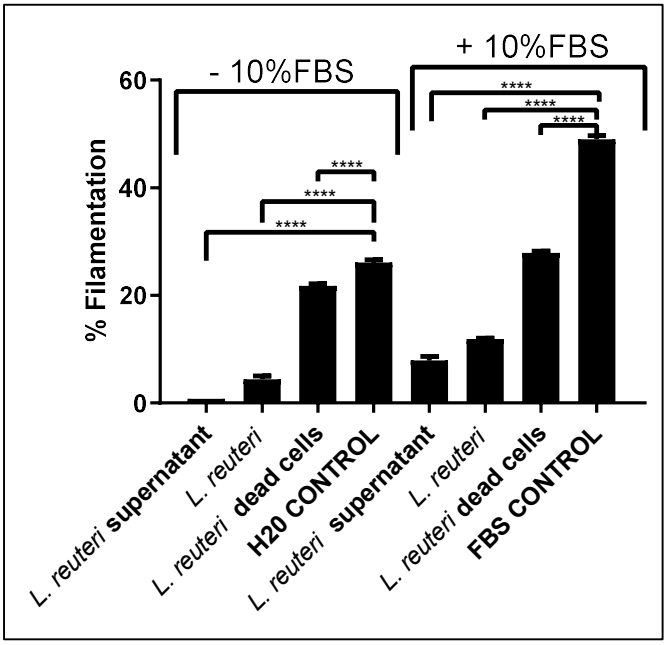

Figure 28 - Filamentation of *C. albicans* after 24h in the presence of different concentration of *L. reuteri* supernatant, *L. reuteri* live cells and *L. reuteri* dead cells. The experiment was performed under MRS medium at 37ºC, 25 rpm in RPMI medium. The results shown were obtained from two independent experiments.

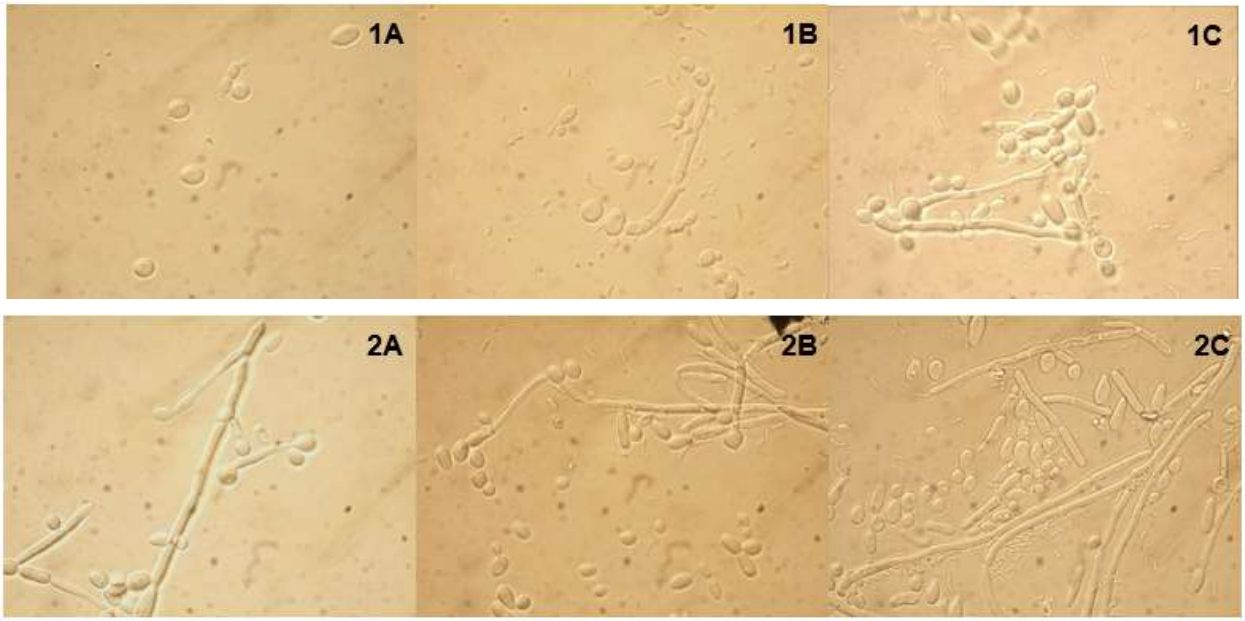

Figure 29 - *C. albicans* without (1A, 1B, 1C) and with (2A, 2B, 2C) FBS, in the presence of *L. reuteri* supernatant (1A/2A), *L. reuteri* live cells (1B/2B) and *L. reuteri* dead cells (1C/2C). Observed by SEM with a magnification of 10000x.

The ability to produce biofilm is another important virulence factor of *Candida* cells (Silva *et al.*, 2009) and the ability to form mixed biofilms with lactobacilli species, including *L. gasseri*, has been reported (Pedro, 2017; Tan *et al.*, 2017; Matsuda *et al.*, 2018; Rossoni *et al.*, 2018). As such, in this work we have examined how the presence of *L. reuteri* would influence the ability of *C. albicans* and *C. glabrata* to form biofilms on the surface of polystyrene (Figure 30). The results obtained show the ability of these cells to form tight mixed-biofilms being observed the establishment of tight interactions among them (Tan *et al.*, 2017).

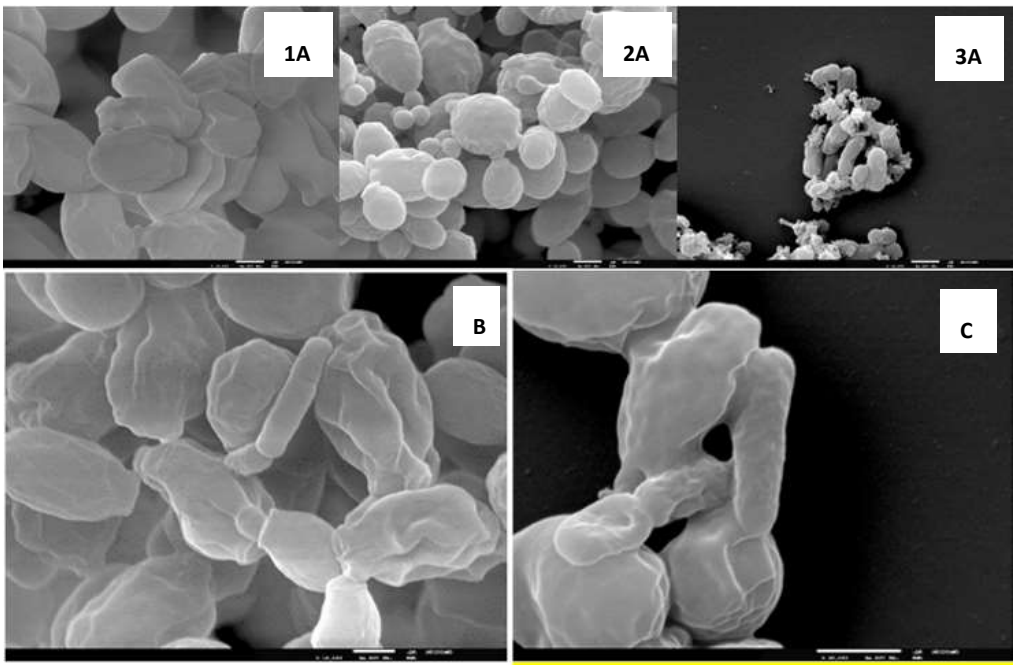

Figure 30 - *C. albicans* (1A), *C. glabrata* (2A) and *L. reuteri* (3A) cells from 0.1 initial OD culture observed by SEM with a magnification of x10000. Co-culture of *L. reuteri* with *C. albicans* (B) (magnification of x10000) and *C. glabrata* (C) (magnification of x20000) from 0.1 initial OD culture observed by SEM.

Effect of *L. reuteri* exopolysaccharide production on the inhibitory effect exerted over *Candida*

Since the exopolysaccharide of other lactobacilli species has been shown to contribute for inhibition of growth and virulence of *Candida* (Tallon, Bressollier and Urdaci, 2003; Badel, Bernardi and Michaud, 2011; Sungur *et al.*, 2017), in this thesis we have examined whether this would also be detected for the exopolysaccharides produced by *L. reuteri*. For this, we have measured the effect of the exopolysaccharide bound to the bacterial cell (EPS-b) or of the one released (EPS-r) to the broth. After purification of these fractions, their effect in inhibition of *C. albicans* and *C. glabrata* growth was tested. Cultivation of *L. reuteri* in 10 mL of the MRS medium led to the isolation of 0.254mg/L of EPS-b and 0.439mg/L of EPS-r. Both the EPS-b and EPS-r showed ability to inhibit growth of *C. albicans* and *C. glabrata*, with the EPS-r being slightly more inhibitory (Figure 31 and Appendix 13).

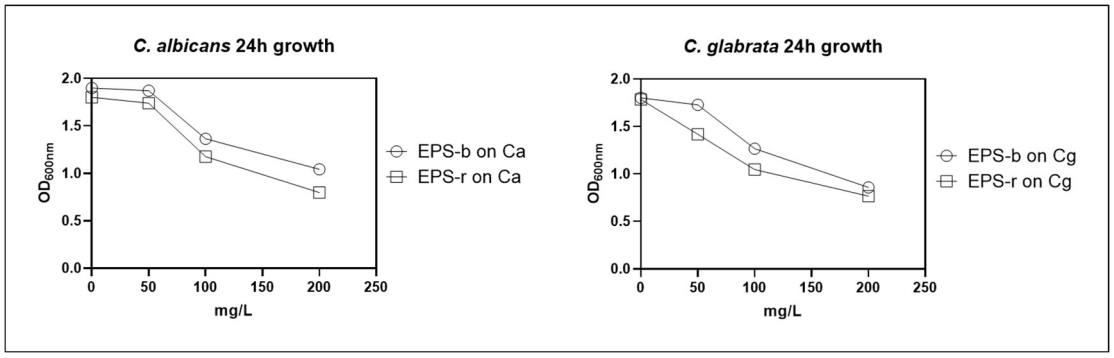

Figure 31 – Comparison between the growth of *C. albicans* (left) and *C. glabrata* (right) under the effect of *L. reuteri* EPS-b and EPS-r.

Effect of *L. reuteri* on *C. albicans* and *C. glabrata* growth in BHI medium

Although the co-culture between *L. reuteri* and *C. albicans* or *C. glabrata* in MRS medium already revealed some potential of the bacterial species to inhibit growth of the two yeasts, the rapid loss of viability observed for the bacteria prompted us to search for another alternative. As such, it was examined the interaction between *L. reuteri* and *C. glabrata* or *C. albicans* during cultivation in BHI medium. The results obtained led to a much more potent inhibitory effect of *L. reuteri* over *C. glabrata*, compared to the effect that was observed during co-cultivation in MRS medium (Figure 32 compared with Figure 24). Significantly, this stronger inhibitory effect of the bacteria over growth of *C. glabrata* was accompanied by a much higher viability of the bacterial species that showed almost no significant loss of viability during the 48h that the experiment last (Figure 32 and Figure 33).

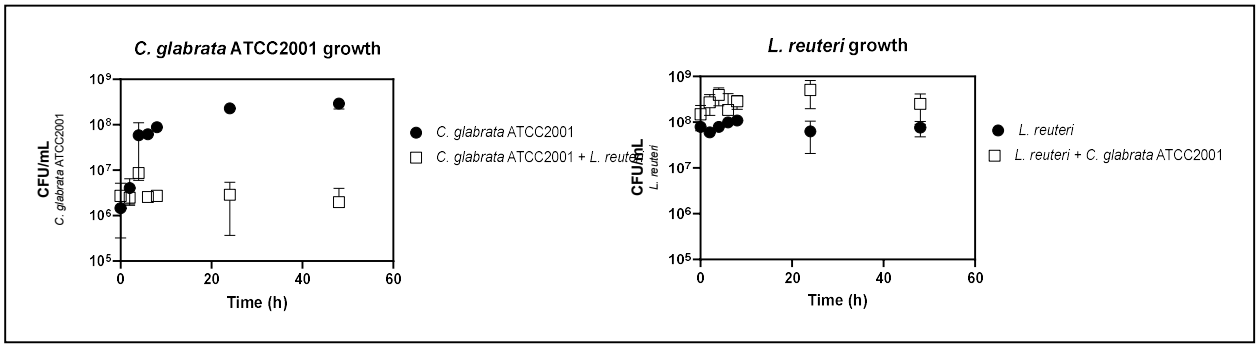

Figure 32 - Growth (CFU/mL) of wild type with *L. reuteri* in BHI medium. The *L. reuteri* growth was determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The results shown were obtained from two independent experiments.

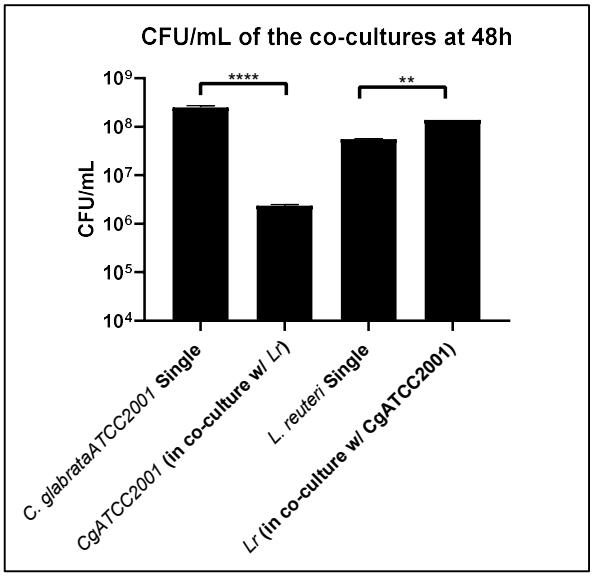

Figure 33 - Viability in CFU/mL at 48h of *C. glabrata* ATCC2001 and *L. reuteri* in co-culture in BHI medium at pH 7 at 37ºC with 100 rpm. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline.

Identical to what happened with *L. gasseri* (Figure 16), *L. reuteri* can inhibit *Candida* growth rate around 61,3%, superior than *L. gasseri* (44,6%). The discrepancy between the two bacterial inhibitions can be due to a faster medium acidification by *L. reuteri,* leading to a more effective supernatant or a maintenance of viability at the end of the co-culture (having a higher inhibition).

Equally to the study with *L. gasseri,* it was tested for *L. reuteri* if the pH-dependent effect was specific of the MRS medium, and for this it was obtained a *L. reuteri* culture supernatant after 48h of incubation in BHI medium. The results obtained demonstrated again a dose-dependent inhibitory effect of this BHI supernatant in inhibiting growth of *C. albicans* and *C. glabrata* (Appendix 15 and Appendix 16), however, in this case a higher effect was observed for the supernatant that was maintained at pH 7 (Figure 34).

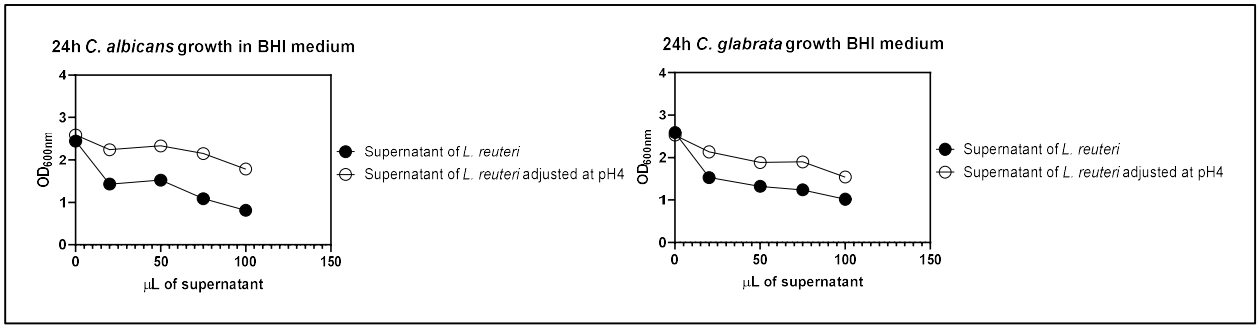

Figure 34 - 24-hour growth curve of *C. albicans* and *C. glabrata* with different concentrations of *L. reuteri* supernatant in BHI medium at pH 4 (adjusted) and 7, at 37ºC with 100 rpm.

Chapter 4 – Discussion

The microbiota present in the human body plays an extremely relevant role in modulating immunity and health, specially by providing protection against a range of relevant pathogens. This thesis is focused on the interactions established between two species of lactobacillus, *L. gasseri*, colonizer of the vaginal tract, and *L. reuteri*, colonizer of the gastrointestinal tract with *C. albicans* and *C. glabrata*. In specific, it was examined the influence of living bacterial cells as well as of cell-free supernatant (CFS) in the physiology and some virulence traits of the two *Candida* species examined. Several studies in this area are already documented, all showing a inhibition by lactobacilli over *Candida* spp. (Khler, Assefa and Reid, 2012; Jørgensen *et al.*, 2017; Mu, Tavella and Luo, 2018). To start it was examined whether the expression of three *C. glabrata* genes found to be up-regulated in the presence of *L. gasseri* had an effect in the equilibrium established between these two species along co-cultivation in MRS medium. The results obtained confirmed a positive beneficial effect for the CgCTA1 gene. The CgCTA1 gene is a catalase gene that *Candida glabrata* harbors, localized in peroxisomes, that is induced by oxidative stress and carbon source availability. In starvation glucose conditions and with hydrogen peroxide stress, the cells displayed a continuous increase of CgCTA1 expression (Roetzer *et al.*, 2010). As this gene protects against oxidative stress and glucose starvation, and in under the conditions tested the H_2O_2 concentration was low, this gene will not be very relevant on the interactions studied. Thus, in order to understand its role and how this gene can protect against oxidative stress it would be interesting to test the CgCTA1 gene in co-culture with *Lactobacillus* species, but at higher H_2O_2 conditions, and compare it with these present results.

After this, it was focused a better study of what could be the factors underlying the inhibitory effect exerted by *L. gasseri* culture supernatants over growth of *C. albicans* or *C. glabrata*, with a particular emphasis on what could be the role played by lactic acid. The results obtained do not support the idea that lactic acid is the inhibitory product underlying the observed toxic effect since concentrations identical to those present in the bacterial supernatant did not produce a significant inhibitory effect on growth of the two yeast species. In previous studies, the independence of yeast growth had been proven in the presence or absence of lactic acid (Lourenço *et al.*, 2019). The fact that the inhibitory effect was much stronger when the pH of the supernatant was not adjusted (and remained at the acidic value of 4) suggests that other acid molecules could be present and could mediate this effect, disregarding lactic acid. A more thorough metabolomic characterization of this bacterial supernatant and how its composition changes along the cultivation time of *L. gasseri* in MRS has to be performed to better clarify this. Thus, it can be interesting to study the *Lactobacillus* secretome in order to analyze the proteins present in the supernatant in single culture and in co-culture with *Candida*. The inhibitory effect of *Lactobacillus* also occurred in the BHI medium, being more eminent in pH 7, contrary to what happens in MRS. This is probably due to the composition of this medium, which being composed with blood, could trigger greater inhibition in bacteria. In the future, it would be interesting deeper studies in this medium and understand why the inhibition here

is given in higher pHs. One hypothesis would be that in higher pHs, inhibition mechanisms do not pass through acids but by another existing compound that is induced to be produced by the presence of blood.

The extension of the studies undertaken in the laboratory until so far to examine the intestinal species *L. reuteri* was another of the accomplished objectives of this thesis. In this thesis it is demonstrated that this intestinal species has a strong potential to inhibit growth of *C. albicans* and *C. glabrata*, above the one exhibited by the vaginal species *L. gasseri*, especially when cultivated in BHI medium. This superior inhibitory effect may be due the production of EPS, which have already been shown to inhibit inflammation factors released by pathogens and decreased its adherence (Matsubara et al. 2016). Although several studies have already reported EPS effects on *Candida* from other *Lactobacillus* species (Allonsius et al. 2017; Jørgensen et al. 2017) it would be interesting to infer the real inhibiting role of EPS, exploring the genes involved in the production of EPS and analyzing if there is any altered expression in that genes when in co-culture.

The virulence of yeasts has also been tested, namely the filament capacity and the formation of biofilms. *Candida* albicans is a dimorphic fungus that manages to pass in a planktonic form to hyphae in order to increase colonization and pathogenicity. Filament capacity was inhibited in the presence of *Lactobacillus reuteri*, the same was observed by other species of *Lactobacillus* (Matsubara et al. 2016; Tan et al. 2017; Pedro 2017). This inhibition was higher when incubated with the supernatant and it is believed to be due to modulation in genetic expression in *Candida* (Matsuda et al. 2018). According to other studies, but with vaginal *Lactobacillus* species, these produce antifungal compounds that can reduce the infection of *C. albicans* leading to inhibition of growth and hyphal induction (Jang et al. 2019). On the other hand, there is in fact the formation of mix biofilms between bacteria and yeasts and it is believed that there is even direct contact between them, leading to inhibition of the last. Previously studies report a decrease in the formation of *Candida* spp. biofilms, when inoculated with supernatants of *Lactobacillus* species, derived from some type of exometabolites produced by bacteria (Tan et al. 2017).

In conclusion, the species of *Lactobacillus* studied in this dissertation came to demonstrate inhibition of yeast growth and proliferation, mainly *L. reuteri* showing a more effective supernatant. The explanation of the inhibition of the supernatant still unknown but is believed to be a set of several factors, acting in synergy and not one compound in particular. Finally, in order to complement this study, it would be interesting in future experiments use vaginal and intestinal cells models and use clinical isolates of *Candida* species.

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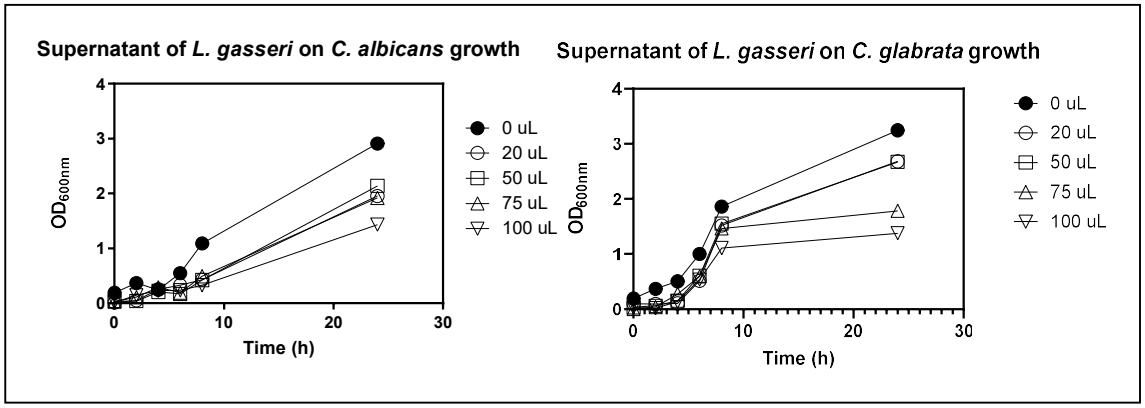
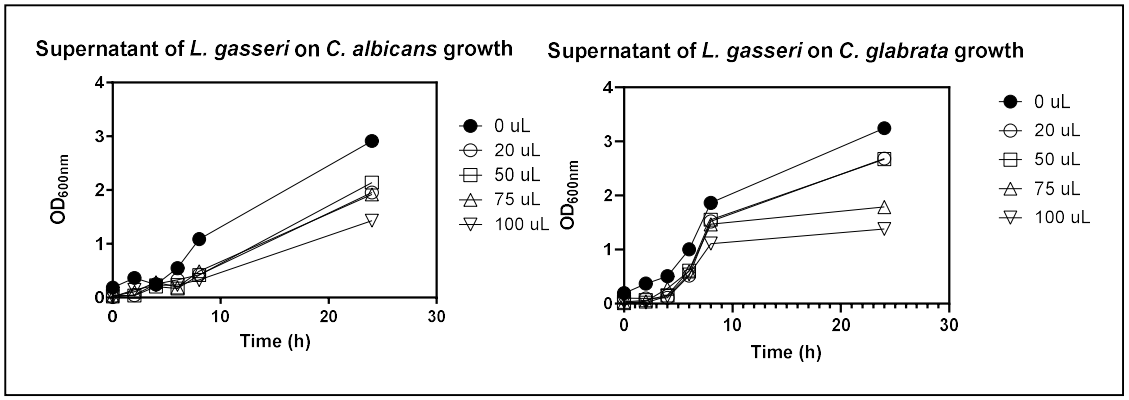
Appendix

	Growth rate	
	single	**w/ *L. gasseri***
***C. glabrata* ATCC2001**	0.2714 ± 0.07845	0.1972 ± 0.0038
CgCTA1	0.1805 ± 0.0004	0.1681 ± 0.03955
CAGL0K04279g	0.1826 ± 0.02155	0.1333 ± 0.0106
CAGL0E03498g	0.1728 ± 0.015378	0.1336 ± 0.04052

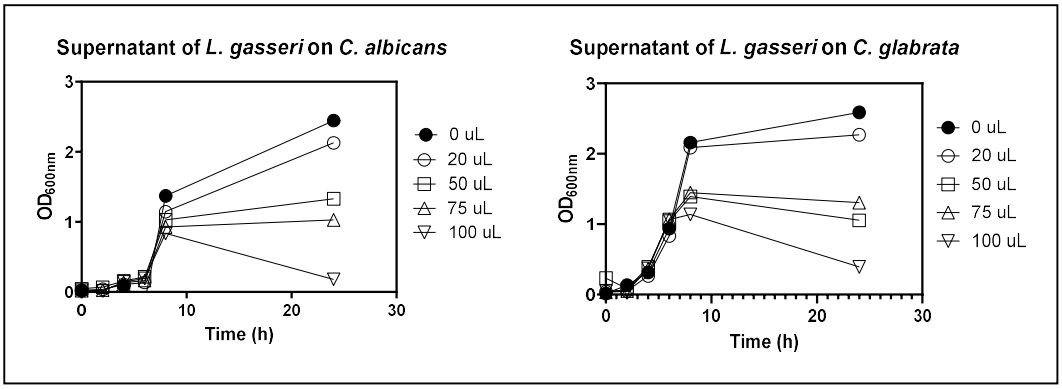
Appendix 1 - Growth rates of *C. glabrata*, control (*C. glabrata* ATCC2001) and mutants (CgCTA1, CAGL0K04279g, CAGL0E03498g) in single and co-culture at 37ºC, 100rpm and MRS medium. The results shown were obtained from three independent experiments



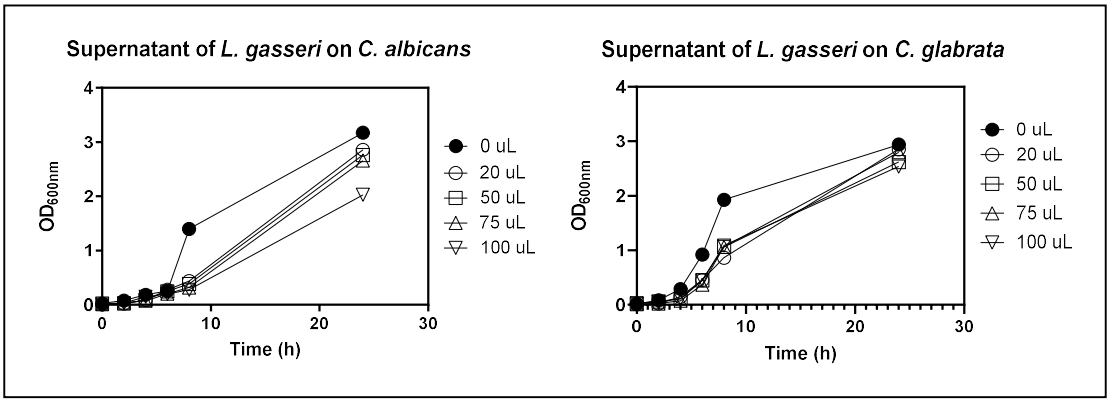
Appendix 2 - Growth curve of *C. albicans* (left) and *C. glabrata* (right) with different concentrations of *L. gasseri* supernatant in MRS medium at pH 4, at 37ºC with 100 rpm (normal condition).



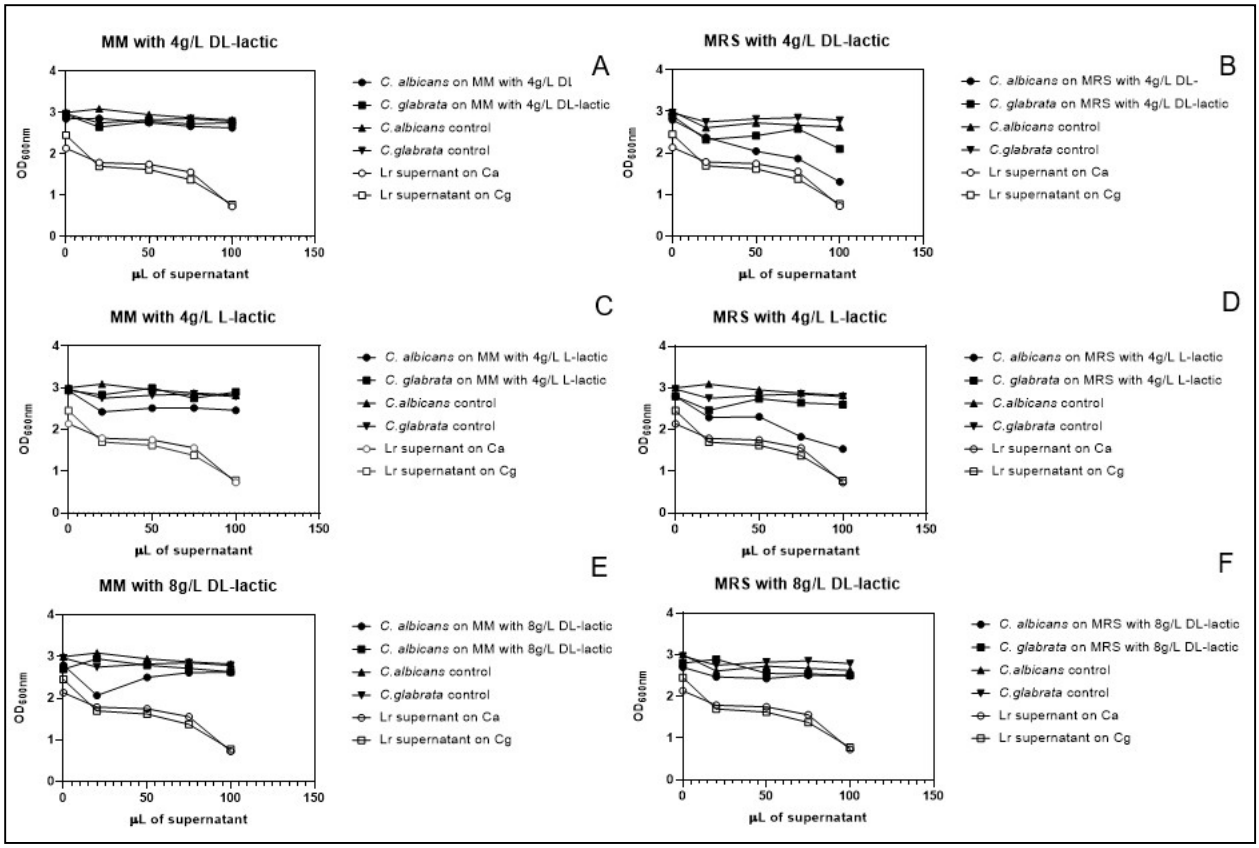
Appendix 3 - Growth curve of *C. albicans* (left) and *C. glabrata* (right) with different concentrations of *L. gasseri* supernatant in MRS medium at pH 7 at 37ºC with 100 rpm.



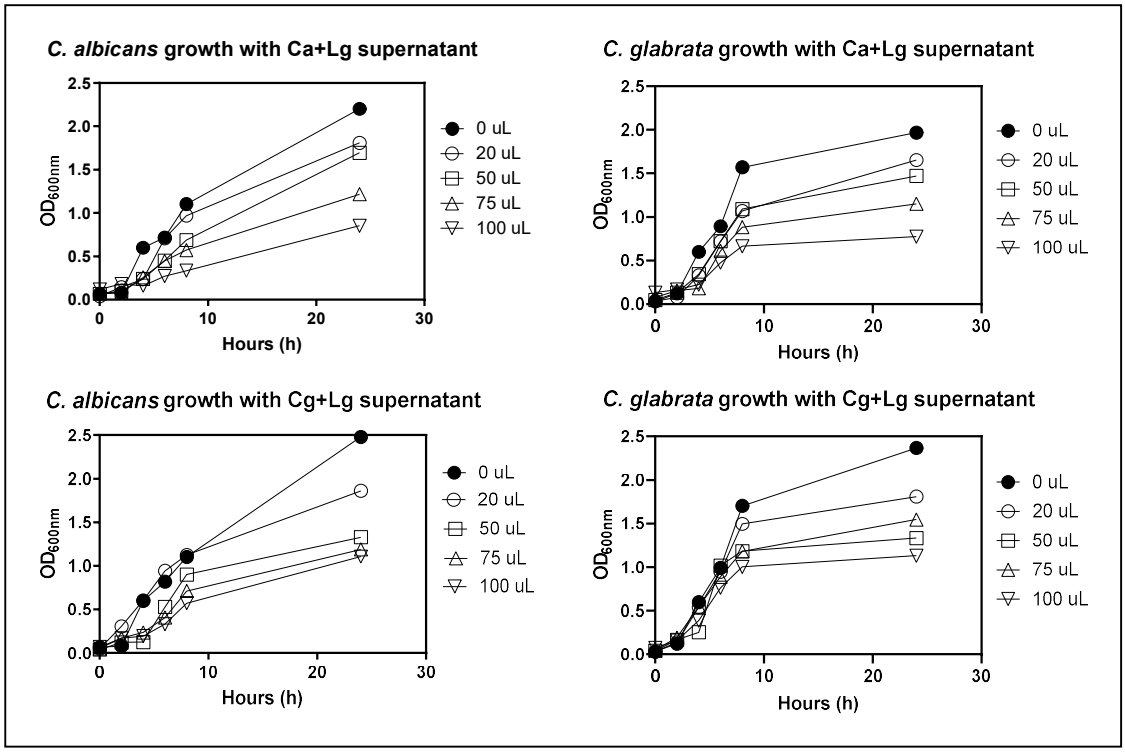
Appendix 4 - Growth curve of *C. albicans* (left) and *C. glabrata* (right) with different concentrations of *L. gasseri* supernatant in BHI medium at pH 7 at 37ºC with 100 rpm.



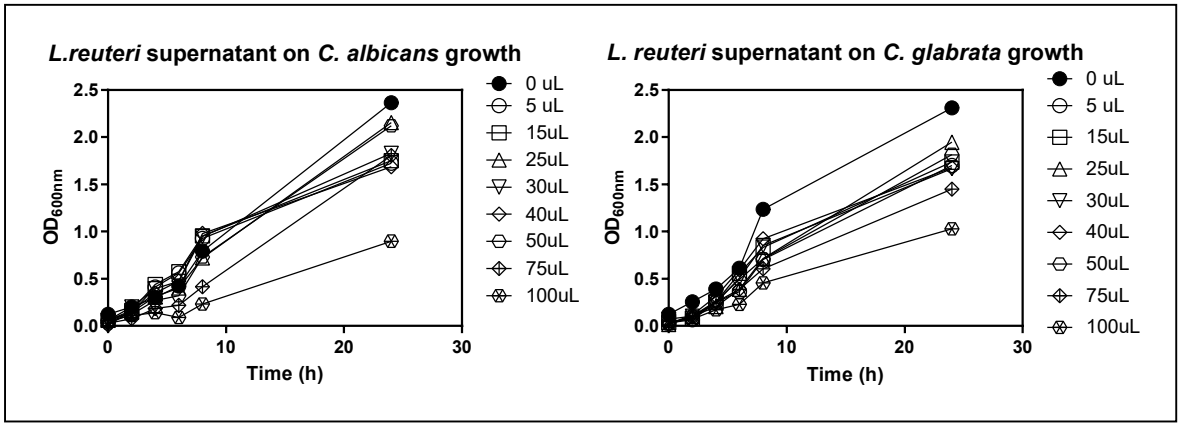
Appendix 5 - Growth curve of *C. albicans* (left) and *C. glabrata* (right) with different concentrations of *L. gasseri* supernatant in BHI medium at pH 4 at 37ºC with 100 rpm.



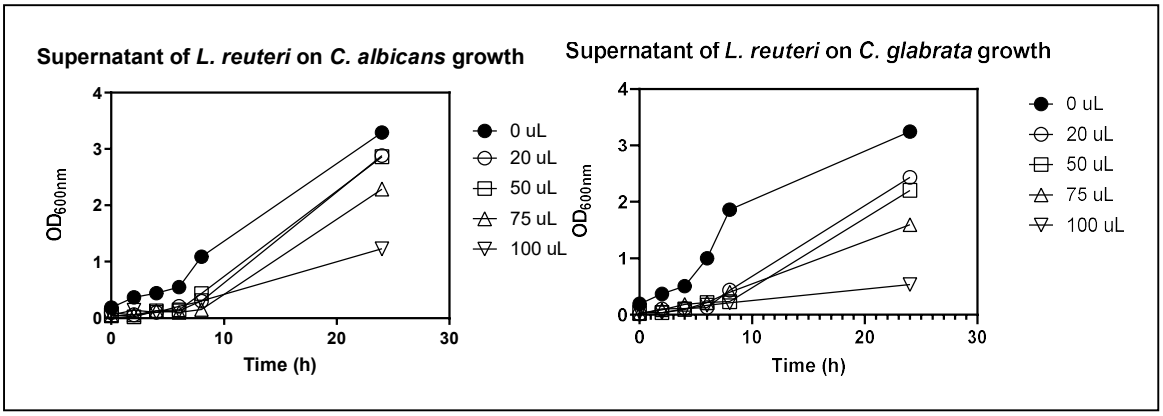
Appendix 6 - Growth curve of *C. albicans* and *C. glabrata* in different mediums with different concentrations of lactic acid. (A) Growth curve of *C. albicans* and *C. glabrata* in minimal medium with 4g/L of DL-lactic acid. (B) Growth curve of *C. albicans* and *C. glabrata* in MRS medium with 4g/L of DL-lactic acid. (C) Growth curve of *C. albicans* and *C. glabrata* in minimal medium with 4g/L of L-lactic acid. (D) Growth curve of *C. albicans* and *C. glabrata* in MRS medium with 4g/L of L-lactic acid. (E) Growth curve of *C. albicans* and *C. glabrata* in minimal medium with 8g/L of DL-lactic acid. (F) Growth curve of *C. albicans* and *C. glabrata* in MRS medium with 8g/L of DL-lactic acid.



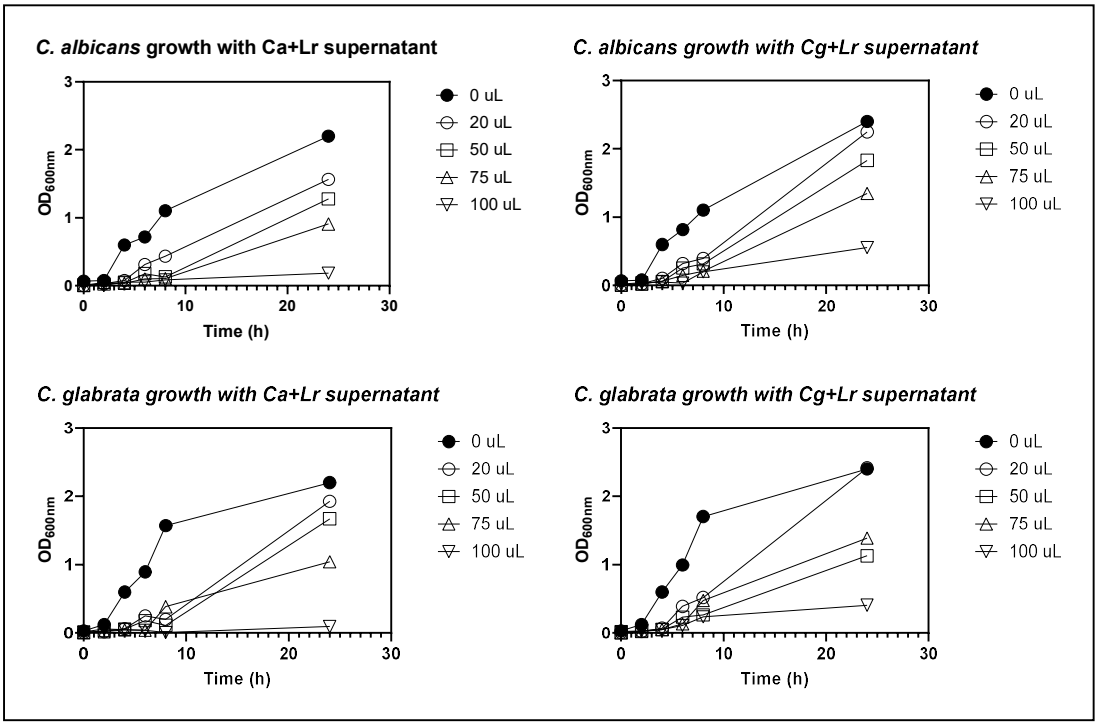
Appendix 7 - Growth curve of *C. albicans* and *C. glabrata* with different concentrations of a co-culture supernatant (*C. albicans* with *L. gasseri* – Ca+*L. gasseri*, *C. glabrata* with *L. gasseri* – Cg+*L. gasseri*) in MRS medium at pH 4 at 37ºC with 100 rpm.



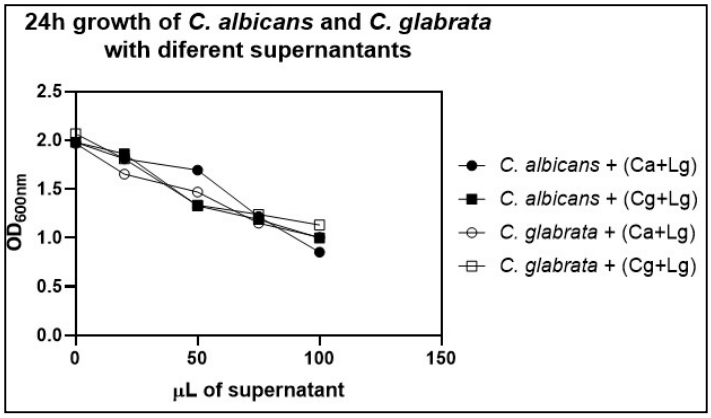
Appendix 8 - Growth curve of *C. albicans* and *C. glabrata* with different concentrations of *L. reuteri* supernatant



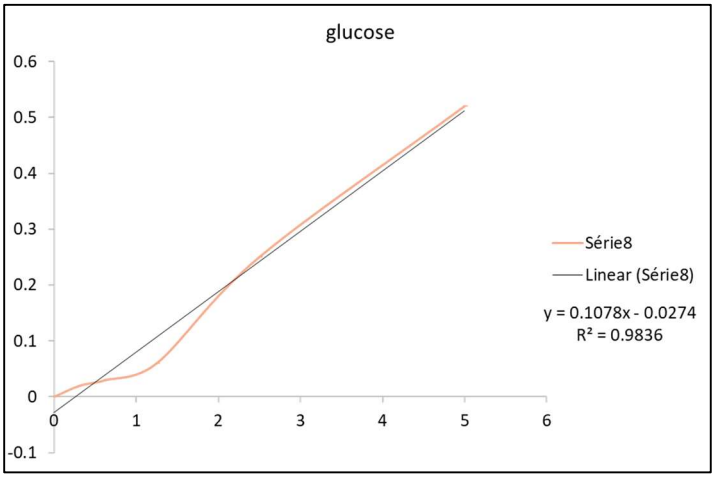
Appendix 9 - Growth curve of *C. albicans* (left) and *C. glabrata* (right) with different concentrations of *L. reuteri* supernatant in MRS medium at pH 7 at 37ºC with 100 rpm.



Appendix 10 - Growth curve of *C. albicans* and *C. glabrata* with different concentrations of a co-culture supernatant (*C. albicans* with *L. reuteri* – Ca+*L. reuteri*, *C. glabrata* with *L. reuteri* – Cg+*L. reuteri*) in MRS medium at pH 4 at 37ºC with 100 rpm.

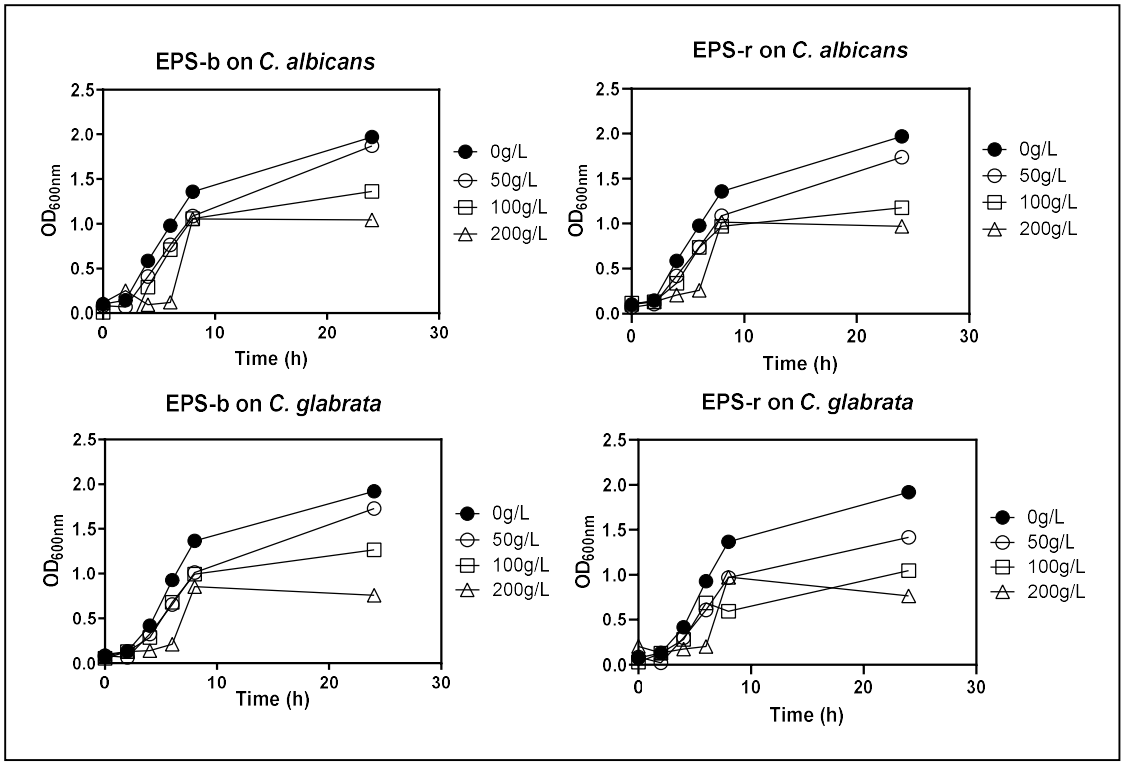


Appendix 11 - 24-hour growth curve of *C. albicans* and *C. glabrata* with different concentrations of a co-cultura supernatant (*C. albicans* with *L. gasseri* – (Ca*Lg*), *C. glabrata* with *L. gasseri* – (Cg*Lg*)) in MRS medium at pH 4 at 37ºC with 100 rpm.



g/L	600nm
0	0
0.3125	0.02
0.625	0.03
1.25	0.06
2.5	0.25
5	0.52
10	1.09
EPS-b	0
EPS-r	0.02
	g/L
EPS-b	0.254174
EPS-r	0.439703

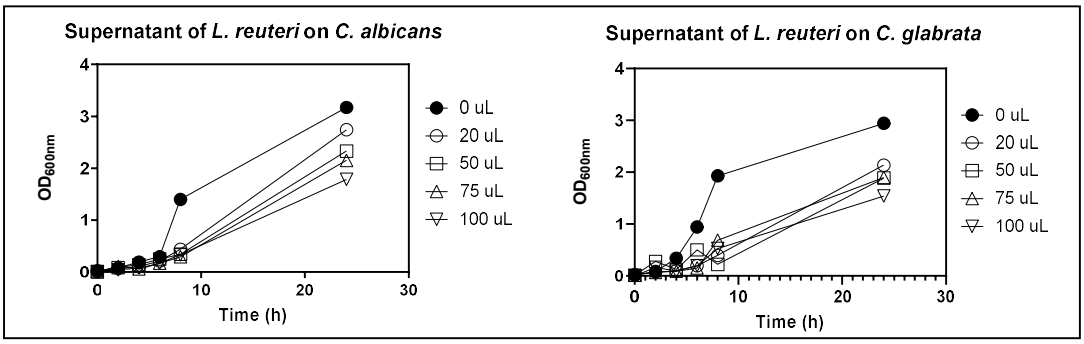
Appendix 12 - EPS Calibration curve with glucose



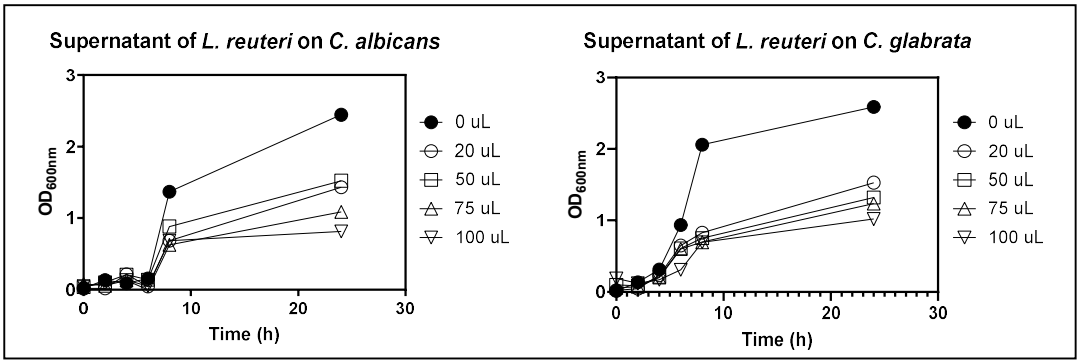
Appendix 13 - Growth curve of *C. albicans* and *C. glabrata* with different concentrations of EPS-b and EPS-r from *L. reuteri* supernatant.

Culture	Growth rate (h^{-1})	
	***Cg* ATCC2001**	***L. reuteri***
L. reuteri	-	0.32
C. glabrata ATCC2001	0.42	-
*Cg*ATCC2001+ *L. reuteri*	0.16	0.14

Appendix 14 - Growth rate of *C. albicans* and *C. glabrata* when co-cultured with *L. reuteri*. Where each result is derived from an average value.



Appendix 15 - Growth curve of *C. albicans* (left) and *C. glabrata* (right) with different concentrations of *L. reuteri* supernatant in BHI medium at pH 4 (adjusted) at 37ºC with 100 rpm.



Appendix 16 - Growth curve of *C. albicans* (left) and *C. glabrata* (right) with different concentrations of *L. reuteri* supernatant in BHI medium at pH 7 at 37ºC with 100 rpm.