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Study of the interaction between vaginal Lactobacilli, *Candida albicans* and *Candida glabrata*: from physiological aspects to transcriptomic analyses

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Abstract

In order to successfully colonize the vaginal niche *C. glabrata* and *C. albicans* have to face multiple environmental insults, which include, among others, the presence of a commensal bacterial microflora that produce several substances that can inhibit *Candida* growth. Metagenomic analyses have revealed that Lactobacilli are predominant in the vaginal microflora and a reduction in the abundance of these bacteria is associated with a higher risk of developing infections. Although the anti-candicidal effect of several Lactobacilli has been shown, these results were obtained *in vitro* and using species other than those indigenous to the vaginal tract. In this work the effect of *L. gasseri* and *L. jensenii*, two of the most abundant species in the vaginal tract, on the physiology and virulence of *C. albicans* and *C. glabrata* were studied. The results obtained showed that exposure to the two bacteria reduces the growth rate of the two *Candida* species, with a higher inhibitory effect registered for *L. jensenii* and *C. glabrata* demonstrating a higher susceptibility. The viability of *C. albicans* and *C. glabrata* cells after 48h of co-culture in the presence of the bacteria was also reduced, a more prominent effect being registered for *C. glabrata*. The presence of *L. gasseri* was also found to reduce filamentation and to influence biofilm formation in *C. albicans*, two key virulence traits of this species. Similar inhibitory effects in growth, biofilm formation and filamentation were also obtained when *C. albicans* and *C. glabrata* cells were exposed to a culture supernatant of *L. gasseri* cells.

To obtain further insights into the responses of *C. glabrata* to the presence of *L. gasseri* a RNA-seq analysis was performed after 2 and 8 hours of co-culture, using as a comparative platform genomic expression of the two species in single-culture. The more prominent alterations in genomic expression were found after 8h of co-culture being registered 638 *C. glabrata* differentially expressed genes (429 up-regulated and 209 down-regulated) and 204 *L. gasseri* genes. These results open the door to a better understanding of the interference between lactobacilii and *Candida* in the vaginal tract fostering the development of new non-conventional therapeutic approaches.

Keywords: *C. glabrata*, *C. albicans*, *L. gasseri, L. jensenii*, lactobacilii-*Candida* interference, RNA-seq

Resumo

Para que a colonização do nicho vaginal seja feita com sucesso, *C. glabrata* e *C. albicans* têm que superar vários stresses ambientais, os quais incluem, dentro de outros, a presença de outras bactérias comensais da microflora que produzem diversas substâncias que podem inibir o crescimento de espécies de *Candida*. Análises metagenômicas revelam que Lactobacilli são residentes predominantes da microflora vaginal e que a redução da sua abundancia está associada a um maior risco de desenvolver infeções. Embora o efeito anti-*Candida* de diversos Lactobacilli tenha já sido demonstrada, tais resultados foram obtidos *in vitro* e usando outras espécies que não aquelas indígenas do trato vaginal. Neste trabalho é estudado o efeito de *L. gasseri* e *L. jensenii*, duas das espécies mais abundantes do trato vaginal, sobre a fisiologia e virulência de *C. albicans* e *C. glabrata*. Os resultados obtidos mostram que a exposição a estas duas bactérias leva à redução da taxa de crescimento das duas espécies de cândida, com maior inibição para *L. jensenii* e *C. glabrata*, demonstrando assim uma maior sustentabilidade. A viabilidade celular de *C. albicans* e *C. glabrata* ao fim de 48h de crescimento em co-cultura também foi reduzida, com maior incidência em *C. glabrata*. A presença de *L. gasseri* também inibiu a filamentação e influenciou a formação de biofilme de *C. albicans*, dois importantes aspetos de virulência nestas espécies. Foram obtidos resultados inibitórios semelhantes sobre o efeito do crescimento, formação de biolfim e filamentação quando *C. albicans* e *C. glabrata* foram expostas a sobrenadantes da cultura de *L. gasseri*.

Para obter mais informações sobre as respostas de *C. glabrata* à presença de *L. gasseri* foi realizada uma analise de sequenciação de RNA (RNA-seq) ao fim de 2 e 8 horas de crescimento das co-culturas, usando como plataforma comparativa a expressão genómica das duas espécies em cultura única. As alterações genómicas mais proeminentes foram registadas ao fim das 8h de crescimento da co-cultura, registando-se 638 genes de *C. glabrata* diferentemente expressos (429 sobre expressados e 209 sob expressados) e 204 genes de *L. gasseri*. A deleção do fator de transcrição *CgHaa1* demonstrou um aumento significativo da susceptibilidade de *C. glabrata* a *L. gasseri*, sendo identificados pelo menos 10 genes de *C. glabrata* sobre expressos que foram já descritos como genes ativados por *CgHaa1*

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Abbreviations

VVC	**Vulvovaginal Candidiasis**
RVVC	**Recurrent vulvovaginal candidiasis**
VMB	**Vaginal Microbiota**
RNA	**Ribonucleic acid**
RPMI	**Roswell Park Memorial Institute medium**
rpm	**Rotations per min ute**
OD	**Optical Density**
CFU	**Colony forming unit**
MFS	**Major facilitator Superfamily**
MMB	**Minimum Medium broth**
MRS	**De Man, Rogosa and Sharpe**
YPD	**Yeast extract Peptone Dextrose**
HPLC	**High Performance Liquid Chromatography**
FPKM	**Fragments Per Kilobase of transcript per Million mapped reads**
MA	**Bland–Altman**
DEG	**Differently Expressed Genes**

Introduction

Overview

Advances in high-throughput sequencing and bioinformatics are just beginning to reveal the breadth, depth, and diversity of the fungal world in humans and throughout the environment. Nobody is fungus-free. Humans have a fungal microbiome (usually called mycobiome) present in many niches including the genito-urinary [1,2,3] the gastrointestinal [4], the respiratory[5] tracts, as well as the oral cavity[6], the scalp or the skin[7]. Among those fungal species that are known to colonize humans as commensals are species of the *Candida* genus namely *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei*[8]. These yeasts are commensal in healthy individuals, however, under certain conditions the commensal populations may overgrow inducing superficial infections more frequent in the oral or in the vaginal tract. In more severe cases, *Candida* spp. can also give rise to disseminated mycoses, in which the yeasts cross the bloodstream and may colonize any major organ[9,10]. Till date, it is not totally clear on whether or not the commensal populations are able to cause invasive infections, although it is generally accepted that they will have the potential to do so. There are several underlying factors in the host for the development of candidiasis including physical insults to the mucosa, disruption of the indigenous microbiota by the use of antimicrobials, expression of specific virulence factors and access to site by excessive number of pathogens[11,12]. Pathogenic traits of *Candida* species include a number of virulence factors, such as the ability to evade host defences, adherence, biofilm formation and the production of tissue-damaging hydrolytic enzymes including proteases, phospholipases and haemolysin[13].

To colonize human niches *Candida* cells have to overcome multiple challenges such as the immune system, variations in pH and in redox potential, differences in the panoply of nutrients available (e.g. iron, zinc or oxygen[14]) and also the presence of a competing microbiota[15]. Lactobacilli are among those species more often found to co-colonize niches with *Candida* spp being present at least in the gastrointestinal and urogenital tracts[16]. In fact, in the vaginal tract the microbiota has been found to be dominated by *Lactobacillus* species[17] out of which *L. acidophilus*, *L. crispatus*, *L. jensenii* and *L. gasseri* are usually the more abundant species[18]. The presence of lactic acid bacteria prevents the overgrowth of vaginal pathogens, including of *Candida* spp, through mechanisms that are yet uncharacterized. Nevertheless, it has been considered that the production of hydrogen peroxide, bacteriocins and the pH alteration of the environment caused by the production of lactic acid are mechanisms that could eventually restrain the overgrowth of vaginal pathogens, including of *Candida* spp[19]. Consistently, several *in vitro* studies have demonstrated the anti-candicidal effect of *Lactobacillus* by positively modulating the host immune system[20], by competing for nutrients and adhesion sites, by producing bacteriocines[21] and by producing organic acids as the result of metabolic activity[22]. In most cases this had been performed using species other than those that are known to be present in the vaginal or in the gastro-intestinal tracts. Lactobacilli are considered probiotics, “live microorganisms which when administrated in adequate amounts confers a health benefit on the

host"[23,24] and in that sense, the possibility of using them as eventual vehicles for the treatment of candidiasis could be of high interest. This thesis is focused on the role of *L. gasseri* and *L. jensenii*, two indigenous species of the vaginal tract, as modulators of the physiology and virulence of *C. albicans* and *C. glabrata*. Besides assessing the effect of the bacteria in growth and virulence traits (e.g. filamentation or biofilm formation) of the two yeasts species in co-culture settings, it has also been briefly studied the effect of the bacteria in genomic expression of *C. glabrata* using RNA-seq.

Vulvovaginal Candidiasis (VVC)

Vulvovaginal candidiasis (VVC) is a mucosal infection of the vaginal tract caused by *Candida* species. *C. albicans* is the causative agent of VVC in approximately 85 to 90% of patients. The rest of the cases are attributable to non-*C. albicans Candida* species, the most common of which are *C. glabrata* and *C. tropicalis*[25]. About 75% of all women are estimated to experience an episode of *Candida* vaginitis in their lifetime[26], with about 13% to 19% of VVC patients suffering from repeated occurrences of this infection, a condition known as recurrent vulvovaginal candidiasis (RVVC)[27]. Vulvovaginal candidiasis is then classified as either sporadic or recurrent on the basis of episodic frequency. It is clinically imperative to distinguish between sporadic and recurrent infections not only to understand the pathogenesis of each but also to formulate specific disease management strategies[28]. Uncomplicated vulvovaginal candidiasis refers to sporadic events. In contrast, complicated vulvovaginal candidiasis refers to clinical situations in which drugs are required to achieve a cure[29]. VVC is rapidly treated by drugs that remove or control the risk factors and thus it remains for most women an infrequent experience. RVVC is a much more serious clinical condition due to the recurrences of symptoms and for its refractoriness to successful treatment[30]. Symptoms include vulvovaginal pruritus, irritation, soreness, dyspareunia, vaginal discharge, vulva erythema, edema, excoriation, and fissure formation[31]. A no malodorous clumpy white discharge is suggestive of VVC but is extremely nonspecific[32]. VVC is treated with various antifungals drugs like fluconazole for *C. albicans* and boric acid for *C. glabrata*, since the latter is much less susceptible to azole antifungals than *C. albicans*. Other treatments for VVC include the nystatin, amphotericin B and clotrimazole or systemic agents such as ketoconazole or itraconazole[33]. Empirically, the ingestion of probiotics has also been pinpointed as a possible strategy to overcome VVC[34].

The role of vaginal microbiota as probiotic

In the last decade, phylogenetic analyses of vaginal samples (mostly bacterial 16S ribosomal RNA gene sequencing) have shown that bacterial communities in the vagina are more complex than previously thought[35]. The microorganisms that compose the vaginal microbiota fluctuate with women's age, hormonal and immune status, sexual activities, use of medications, mode of contraception, and exposure to a variety of vaginal products[36,37]. Despite this, in almost all studies performed it has been observed a predominance of Lactobacilli and alterations in this can cause symptomatic conditions[35]. The most frequently isolated species are *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners*[38] although the *Lactobacillus* species found in vaginal microbiota can fluctuate from an healthy VMB or a case of dysbiosis VMB (Figure 1)[38].

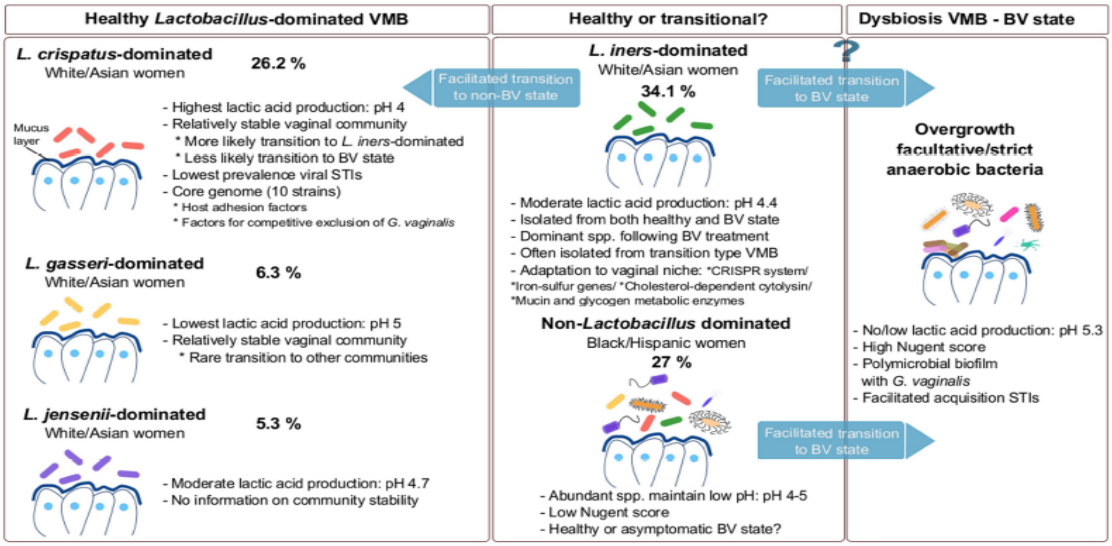

Figure 1 - Composition of VMB during healthy and dysbiotic states[38]

In the following section it is described a bit of what is known concerning physiological and genomic aspects of *L. gasseri* and *L. jensenii* species which were most focused in this work.

L. gasseri and *L. jensenii*: a brief overview on physiology and anti-*Candida* potential

The morphology of *L. gasseri* cells is rod with rounded ends that can appear as a single cell or in a chain morphology. *L gasseri* cells are non-mobile and non-flagellated. Growth of *L. gasseri* is greatly enhanced by anaerobiosis and in environments with a low concentration of CO_2. These cells can ferment glucose, fructose, sucrose, cellobiose, and salicin[39], almost always using a homofermentative carbon metabolism that leads to the production of lactic acid. Optimal temperature for growth ranges from 35 to 38 degrees, with no growth being detected below 22 degrees[39]. Regarding pH tolerance, *L. gasseri* is able to growth at very acidic environments (pH 2)[40]. This species has be found in various niches in the human body including the mouth, intestine, faeces, to the vagina of juvenile or adult women[39]. The genome of *L. gasserii* is around 1.894.360 bp in size, it does not exhibit plasmids and is predicted to encode 1.810 genes, these coding sequences accounting for 88% of the overall genome[41]. The GC content is 35.3%, in line with the reported GC content of its closest relatives, *L. johnsonii* NCC 533 (34%) and *L. acidophilus* NCFM (34%)[41].Thirteen putative transposon or transposon-related genes were identified in the genome sequence of *L. gasseri*, this number being close to those reported for *L. acidophilus* and *L. johnsonii. L. gasseri* contains 75 tRNA genes, in accordance with the number of tRNA genes and genome size of Firmicutes[41].

L. jensenii is an anaerobic, gram-positive bacterium that has a thick peptidoglycan wall which results in a rod-shaped aspect. *L. jensenii* optimal growth conditions require the presence of folic acid, vitamin B12, nicotinic acid and calcium pantothenate at 37ºC. Its genome exhibits a GC content of 36.1%, which is within the range of what is observed for other Lactobacilli. *L. jensenii* differs from be other *Lactobacillus* species by hydrolysing arginine, producing only D-lactate and ability to ferment

galactose, esculin, maltose amygdalin, and ribose. It is also not able to ferment lactose, melexitose, mannitol, sorbitol, arabinose, and xylose[42]. *L. jensenii* is responsible for production of acid in the vagina by anaerobic metabolism of glycogen.

The molecular mechanisms used by Lactobacilli to inhibit the over-growth of *Candida* and other vaginal pathogens are not fully described. It was observed that the vaginal microbiota of patients with diagnosed RVVC has been shown to be depleted from Lactobacilli, which further reinforces the idea that bacteria play an important role in maintaining vaginal health[43]. The underlying fungicidal or fungistatic effects of probiotics may involve the production of secondary metabolites with antimicrobial activity, the competition for nutrients and adhesion sites, the stimulation of the immune system[44] and the production of acids, like lactic and acetic acid, that maintain a low pH (<4.5) in the vagina[45]. Also, the development of VVC has been associated with the lack of H_2O_2-producing *Lactobacillus* species[46]. The following Figure 2 represents the possible mechanisms by which *Lactobacillus* can inhibit growth of *C. albicans*. Evidence suggests that the bacterium reduces the adhesion of *C. albicans* to epithelial cells either by (a) outcompeting fungal cells for adhesion sites, such as cellular receptors to which *Lactobacillus* has higher affinity, or (b) by secreting biosurfactants (such as surlactin) that physically decrease fungal binding. Most *Lactobacillus* strains release (c) hydrogen peroxide (H_2O_2) and (d) lactic acid or other fatty acids that inhibit *C. albicans* proliferation and invasive hypha formation. Bacteriocin-like substances (e) produced by *Lactobacillus* can also suppress fungal growth to directly decrease its load[21].

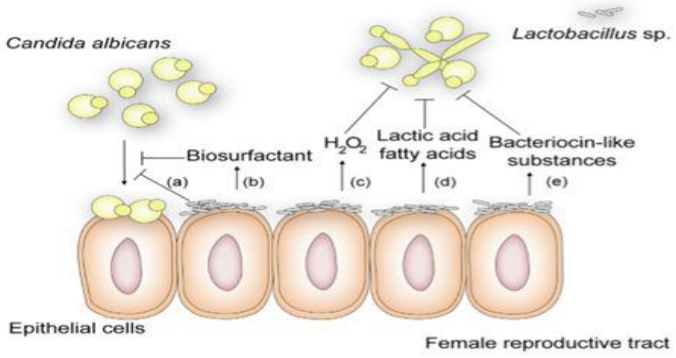

Figure 2 - Representation of the putative mechanisms of inhibition of *C. albicans* by *Lactobacillus* sp[21]

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

As said before, the production of lactic acid and of acetic acids is considered as a possible way by which Lactobacilli can restrain the overgrowth of pathogens through the acidification of the extracellular environment. In Lactobacilli the carbon source is metabolized via homofermentative or heterofermentative pathway. Homofermentation of hexoses is performed via Embden-Meyerhof-Parnas pathway producing lactate[47], while heterofermentation of hexoses is made through the 6-P-gluconate/phosphoketolase pathway producing lactate, acetate/ethanol and CO2[48] (as schematically represented in Figure 3). Pentoses are heterofermentated by 6-phosphogluconate/phosphoketolase. Pentoses can also be fermented by some Lactobacilli by homofermentation[49].

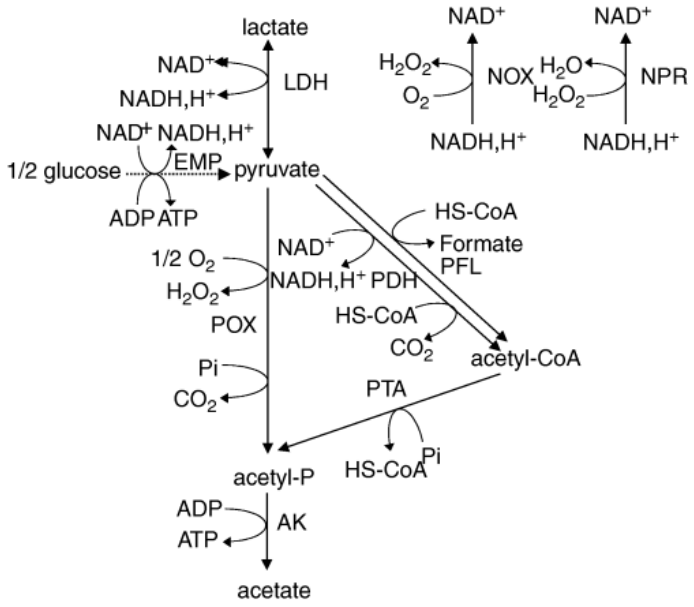

Figure 3 - Lactate and acetate production pathways in *Lactobacillus plantarum*. EMP, Embden–Meyerhof Parnas pathway; LDH, lactate dehydrogenase; POX, pyruvate oxidase; PDH, pyruvate dehydrogenase; PFL, pyruvate formate lyase; PTA, phosphotransacetylase; AK, acetate kinase[50]

The antimicrobial effect of weak acids results from the fact that when present in aqueous solution these molecules partly dissociate leading to a dynamic equilibrium between the undissociated acid form and the anions/protons. At a pH below the acid pKa value, this equilibrium favours the undissociated acid form which is lipid-soluble and therefore able to penetrate the lipid bilayer of the plasma membrane by simple diffusion dissociating directly in the cytosol (which has a pH close to neutrality)[51]. In this sense, the antimicrobial potential of carboxylic acids is largely determined by their pKa and also hydrophobicity[52]. At the acidic environment of vaginal pH (pH 3.6-4.5, depending on dominant *Lactobacillus* spp.)[53] approximately 80% of acetic acid and 42% of lactic acid (values estimated for a pH of 4.0) are expected to be found in their undissociated form (pka acetic acid=4.76; pka lactic acid=3.86). Once in the near-neutral pH cytosol the organic acid will dissociate leading to the consequent accumulation of protons and of the negatively charged counter-ion[52,54]. The molecular mechanisms underlying the adaptive responses of *C. glabrata* to high concentrations of lactic and acetic acid under low pH conditions are still scarce; however extensive knowledge has been obtained in *Saccharomyces cerevisiae*, a species closely related to *C. glabrata*[54].

To offset the increased flux of protons that result from dissociation of the acid and from membrane permeabilization, *S. cerevisiae* cells rely on the activity of two proton pumps, one located in the plasma membrane, the PM-H+-ATPase protein, encoded by the *PMA1* gene, and the other located in the vacuolar membrane, the V-ATPase (Figure 4). Pma1p excretes the exceeding protons to the cell exterior while V-ATPases catalyses their efflux to the lumen of the vacuole. The activity of these two proton pumps counter-acts the dissipation of the plasma and vacuolar membrane, respectively, also contributing for the maintenance of intracellular pH within physiological values. Both these two proton pumps have been described to have a role in yeast response and resistance to acetic and lactic acids[52,55]. Notably, in *C. glabrata* it has been demonstrated that under acetic acid

stress at a low pH (pH 4) there is an up-regulation of the plasma membrane proton pump, consistent with the idea that this is also a mechanism used by this species to cope with the acid-induced intracellular acidification[56]. Due to its negatively charged nature, the counter-ion cannot diffuse across the plasma membrane and therefore its reduction in the intracellular environment relies on the activity of specific inducible transporters. The expression of some of the multidrug resistance efflux pumps from the major facilitator superfamily (MFS), including *CgDTR1*, *CgTPO3* and *CgAQR1*, has been found to increase tolerance of *C. glabrata* cells to acetic acid, although this protective role could only be linked to a presumable effect in transport of the anion in the case of the first two transporters[57–59].

The active expulsion of weak acid anions from the cell interior would be energetically expensive and futile if the undissociated acid could re-enter the cells at a similar rate. Consequently, one of the mechanisms proposed to reduce the diffusion rate of weak acids is the reinforcement of cell wall structure to decrease its porosity[60,61] (Figure 4). Consistently, several genes encoding components of the cell wall (*CTS1, DSE2, EGT2, SCW11* and *SED1*) were found to confer resistance against lactic acid-induced stress[55] in *S. cerevisiae.* From those genes, only two (*EGT2* and *SCW11*) were proved to have a prominent role in cells challenged by inhibitory concentrations of acetic acid[55]. Other genes related to cell wall function were also identified as determinants of resistance to acetic acid including genes involved in the assembly and remodelling of the cell wall structure (e.g. *BPH1, GAS1*, *CWH43)* involved in the synthesis of cell wall polysaccharides (e.g. *FKS1, KRE1*, *CHS1*) and proteins responsible for the mannosylation of proteins to be incorporated in the mannan layer (e.g. *MNN2, MNN9, KTR4, GON7*)[62]. Notably, evidences were recently obtained indicating that *C. glabrata* vaginal isolates found to be highly tolerant to acetic acid exhibit marked differences in the structure of the cell wall structure, this being suggested to result in an important impermeabilization of the cells to the undissociated form of acetic acid[54].

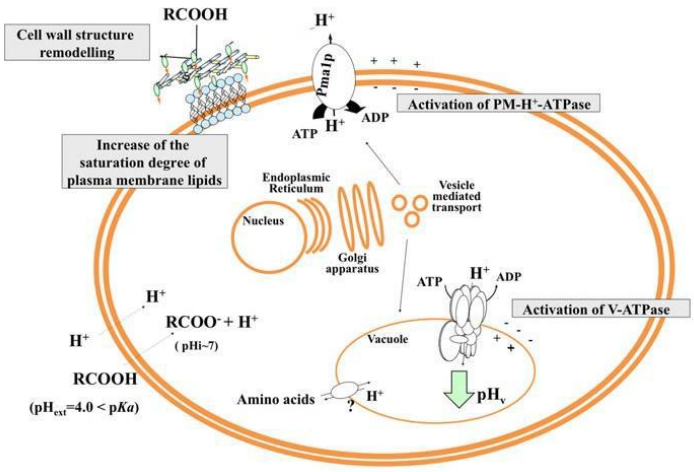

Figure 4 - Adaptive response in yeast cells: weak organic acids ability to cross plasma membrane, intracellular pH recovery and reconfiguration of cellular envelop[52]

The ability to metabolize acetic acid even when glucose is present in the growth medium as also been pinpointed as an important mechanism by which *C. glabrata* cells cope with high concentrations of acetic acid at a low pH[54], this also being an important detoxification mechanism reported in other acetic acid-tolerant species[63]. This observation suggests that unlike *S. cerevisiae*, in *C. glabrata* the presence of glucose does not repress the metabolization of other alternative carbon sources which should contribute to improve metabolic flexibility in the often glucose-deprived niches colonized by *C. glabrata*[54]. Similarly, it has also been shown that *C. albicans* cells are able to consume lactate in the presence of glucose[64].

One very important gene that regulates acetic acid stress tolerance in *C. glabrata* is the transcription factor *CgHaa1*. Transcriptomic analysis showed that *CgHaa1* regulates, directly or indirectly, the expression of about 75% of the genes activated under acetic acid stress; thereby emerging as a critical regulator of *C. glabrata* genomic expression under acetic acid stress[57]. *CgHaa1* expression increased the activity and the expression of the CgPma1 proton pump and contributes to the increasing colonization of vaginal epithelial cells by *C. glabrata*. Two identified *CgHaa1*-activated targets, *CgTPO3* and *CgHSP30*, were described as determinants of *C. glabrata* tolerance to acetic acid[57].

Introduction to the theme of the thesis

This thesis is focused on the effect exerted by two relevant vaginal species of *Lactobacillus*, *L. jensenii* ATCC 25258 and *L. gasseri* ATCC 33323, in the physiology and also in some virulence traits of *C.albicans* and *C. glabrata*. In specific it was implemented a co-culture setting to assess growth of these different species in the presence of each other. The ability of the species to form biofilms when cultivated in the presence of each other, either single-species or involving more than one species, was also examined, as well, the filamentation capacity of *C. albicans*. The co-culture setting developed was also used to examine the ability of vaginal *C. albicans* and *C. glabrata* isolates or of a strain devoid of *CgHaa1* gene, to grow in the presence of *L. jensenii* and *L. gasseri*. At last, the co-culture setting established was used to perform a transcriptomic profiling of *C. glabrata* and *L. gasseri* either when cultivated in single or co-culture aiming to assess how the microbes evolve their response to cope with the presence of each other in the environment. A brief overview on the results obtained in this transcriptomic analysis is herein shown, this being the first time that response of *C. glabrata* to a relevant *Lactobacillus* species present in the vaginal microbiome is addressed.

Materials and methods

1) Strains and Media

The strains used in this work are listed in Table 1

Table 1 - *Candida* and *Lactobacillus* strains studied

Strain name	Description	Source
***C. glabrata* KUE100**	Wild-type strain derived from the CBS138 strain[65]	[57]
***C. albicans* SC5314**	Reference strain; clinical isolate recovered from the blood of an infected patient	-
***C. glabrata* Δhaa1**	KUE100_ΔCAGL0L09339g	[57]
***C. albicans* VG216**	Vaginal clinical isolate	[54]
***C. albicans* VG217**	Vaginal clinical isolate	[54]
***C. albicans* VG485**	Vaginal clinical isolate	[54]
***C. glabrata* BG2**	Vaginal clinical isolate	[54]
***C. glabrata* VG99**	Vaginal clinical isolate	[54]
***C. glabrata* VG281**	Vaginal clinical isolate	[54]
***C. glabrata* VG49**	Vaginal clinical isolate	[54]
***C. glabrata* VG216**	Vaginal clinical isolate	[54]
***L. gasseri* ATCC 33323**	Reference strain	DSMZ
***L. jensenii* ATCC 25258**	Reference strain	DSMZ

All the species were cultivated in MRS medium under microaerophilic conditions. Liquid MRS medium (DSMZ) 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 25^oC was performed. In the case of solid MRS solid, this was acquired from Sigma-Aldrich and was prepared according to manufacturer instruction, 62 g/L, prior to sterilization in autoclave.

RPMI (Roswell Park Memorial Institute) medium, MMB (Minimal medium broth) and YPD (Yeast extract – Peptone – Dextrose) were also used. RPMI (contains, per liter, 10.8g RPMI-1640 synthetic medium (Sigma), 18g glucose (Merck Millipore) and 34,5g of MOPS (3-(N-morpholino) propanesulfonic acid, Sigma). MMB contains, per liter, 1.70 g yeast nitrogen base (YNB) without amino acids and NH4+ (Difco Laboratories, Detroit, Mich.), 2.65 g (NH4)2SO4 (Merck Millipore) and glucose (20 or 10 g/L) (Merck Millipore, Darmstadt, Germany. YPD contains, per liter, 20 g glucose (Merck Millip3ore), 10 g yeast extract (HiMedia Laboratories, Mumbai, India) and 20g peptone (HiMedia Laboratories) and 30% glycerol (v/v) (Merck).

2) Single-species or multi-species cultivation

To assess individual growth of *L. jensenii*, *L. gasseri* and all *Candida* strains in liquid MRS medium at microaerophilic conditions, a pre-inoculum of each species was prepared by inoculating some colonies for 24h in liquid MRS, at 37ºC and using an agitation of 100 rpm. After 24h, the optical density at 600 nm (DO_{600nm}) of these pre-cultures was measured and used to inoculate fresh MRS medium 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, hourly in the case of the *Lactobacillus* species and every two hours in the case of *Candida* species. 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 of cell growth for the first 8h. Lactobacilli were plated in MRS solid plates and incubated for 48h while the *Candida* species were plated in solid YPD. The MRS plates were incubated at 37ºC in a microaerophilic environment using Genbox – Genbag from Biomerieux, while YPD plates were incubated at 30^{o}C under aerophilic conditions.

Co-cultures of *L. jensenii* (or of *L. gasseri*) 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 liquid medium at 37ºC and 100 rpm. After this time, the cells were inoculated in fresh MRS medium aiming to obtain an initial DO_{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 under microaerophilic conditions in Genbox – Genbag from Biomerieux at 37ºC for 48, while the plates used to assess growth of *Candida* were put at 30^{o}C under aerobic conditions.

3) Effect of bacterial supernatant in growth of *Candida*

The supernatant of a 48 hour culture of *L. gasseri* in MRS medium was examined for its potential to inhibit growth of *Candida* in 96-microwell plates. For this, mid-exponential phase cells *C. albicans* and *C. glabrata* cultivated in MMB pH4 (minimum medium broth) were inoculated (at an OD_{600nm} of 0.05) in MMB pH4 (minimum medium broth) supplemented with different amounts of the bacterial supernatant (5,15,25,30,40,50,75 and 100 μL). Each well with *L. gasseri* supernatant was set to 100 μL with or without addition of sterile water, and 100μL cellular suspension was added. Cellular suspension was prepared with an OD_{600nm} of 0.1 in MMB pH4 (2x concentrated). The microplates were incubated at 37ºC at 25 rpm. The OD was measured at 2h, 4h, 6h 8h, and 24h with SPECTROstar^{Nano} from BMG LABTECH, and the growth rate was obtained.

4) Quantification of glucose, ethanol and lactic acid in culture supernatants

To accompany the formation of lactic acid and the consumption of glucose in the single and co-culture settings used samples of the culture supernatants were taken along the growth curves. 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.

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

To assess the effect of a 48h *L. gasseri* culture supernatant or of the live cells on the ability of *C. albicans* cells to trigger filamentation the yeast cells were cultivated in RPMI (pH 4 and pH 7) or MRS. For this, mid-exponential phase cells *C. albicans* cultivated in RPMI (pH4 and pH7) or MRS were: a) inoculated with OD_{600nm} of 0.1 in RPMI (pH4 and pH7) or MRS medium with *L. gasseri* live cells (prior cultivated in MRS medium) in a proportion of 1:4 (yeast:bacteria) for live cells interference in the ability of *C. albicans* cells to trigger filamentation; b) inoculated with OD_{600nm} of 0.1 in RPMI (pH4 and pH7) or MRS in a proportion of 1:2 (fresh medium:*L. gasseri* supernatant) for *L. gasseri* culture supernatant interference in the ability of *C. albicans* cells to trigger filamentation. Also, was performed the same assays with FBS (fetal bovine serum). In these cases, the medium was supplemented with 1:10 FBS. The culture was incubated at 37ºC with 100 rpm. All the observations were obtained with a Zeiss microscope using a 1000x magnification.

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

The effect of the 48h *L. gasserii* culture supernatant (25, 50 or 100 μL) 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 3). The microplates were incubated at 37ºC and 25 rpm for 8h and 24h. After these times, the medium was carefully removed from each well and cells were washed twice with 100μL PBS (Phosphate-buffered saline). Then, prestoBlue reagent was diluted with RPMI pH4 in a proportion of 1:10 (prestoBlue reagent:medium) and 100μL of this solution was added to the cells and incubated for 30 minutes at 37ºC[66]. The OD of the reagent was then measured after a period of incubation of 30 mins. The OD measured was directly proportion of the biofilm produced.

7) Microscopy analysis of single- and multiple-species cultures.

For microscopy analysis by SEM (Scanning Electron Microscopy) of single- and multiple-species cultures an experimental setting similar to the one described above were used with the difference that this was performed in 5 mL polystyerene plates instead of shake flasks. Only a 1:2 proportion between the Lactobacilli and the *Candida* species were used. After 24h of incubation at 37ºC with 100 rpm agitation, the supernatant was removed and the cells adhered to the surface of the

plate were dehydrated using the following protocol: washing with distilled water; washing with 70% ethanol for 10 minutes; washing with 95% ethanol for 10 minutes; and washing with 100% ethanol for 20 minutes. Plates were then dried in a desiccator for 168h and visualized by scanning electron microscope (SEM) using a magnification between 200x and 20000x with a high voltage of 20.0 kV.

8) Transcriptomic analysis

Both single-cultures of *L. gasseri* (0.4 initial OD) and *C. glabrata* (0.1 initial OD) and co-culture (0.4:0.1 bacteria:yeast proportion initial OD) were cultivated, in triplicates, in MRS medium at 37ºC and 100 rpm in a 120 mL shake-flask. Cells were harvested after 2h and 8h of cultivation by centrifugation (6000 rpm, 6 minutes and 4ºC). The obtained cell pellet was frozen at -80ºC until further RNA extraction. RNA extraction and purification were performed using the RiboPure™-Yeast Kit from Invitrogen. Purified RNA from each culture was analysed on a Bioanalyzer to confirm integrity. Six pools were prepared from each time point (2h and 8h) for each single cultures of *L. gasseri*, single cultures of *C. glabrata* and co-cultures. Each pool was then sent and processed by CD genomics for transcriptomic analysis.

Results

Optimization of a co-culture setting using Lactobacilli and *Candida* spp

To establish a system that could be used to study co-cultures of Lactobacilli and *Candida* it was necessary to identify a growth media where all the species involved (*L. gasseri*, *L. jensenii*, *C. albicans* and *C. glabrata)* could grow. For this it was attempted to cultivate the strains (at a temperature of 37ºC and using an agitation rate of 100 rpm) in YPD and MRS growth media. The results obtained showed that the Lactobacilli were not able to grow in YPD, while in MRS all the strains grew, as it can be seen by the growth curves and also by the results of the assessment of the number of colony forming units (Figure 5). Despite this, under the experimental conditions used it was clear that the growth rate of the two Lactobacilli is below the one registered for the two yeast species (Table 2). On the overall the results show that all species can grow in the MRS medium for which it can be concluded that this is an appropriate medium to be used in a co-culture setting.

Table 2 – Growth rate of individual cultures based on optical density at 600_{nm} for the species in studied at 37ºC, 100 rpm in MRS medium

Species	Growth rate (h^{-1})
***L. gasseri* ATCC 33323**	**0.25**
***L. jensenii* ATCC 25258**	**0.19**
***C. albicans* SC5314**	**0.35**
***C. glabrata* KUE100**	**0.31**

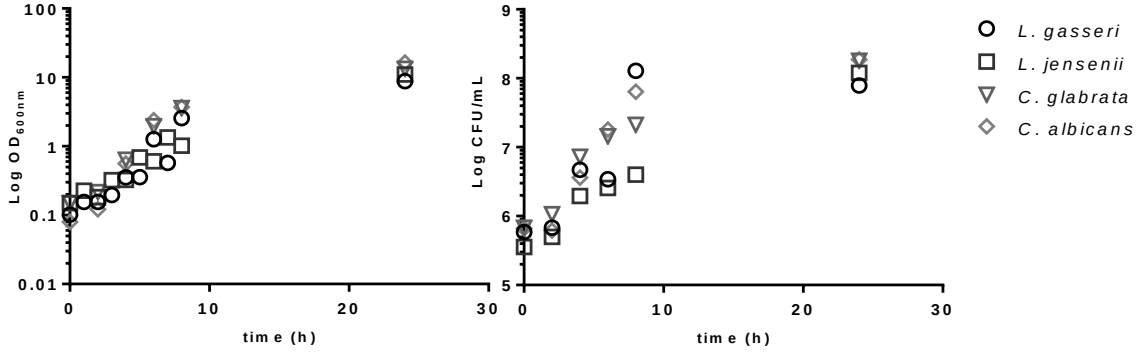

Figure 5 - Growth curve of *C. glabrata*, *C. albicans*, *L. gasseri* and *L. jensenii* in MRS medium at 37ºC with 100 rpm (left) and Log CFU of *C. glabrata* and *C. albicans*, *L. gasseri* and *L. jensenii* cultures in solid YPD and MRS, respectively (right)

A second aspect that had to be optimized was how to assess growth of the two species in a co-culture setting since the increase in the OD could no longer be used. In that sense it was decided to follow growth of the strains based on the increase in the number of CFUs along time. For this it was necessary to establish a system that could specifically inhibit one of the species involved in the co-culture. In this context, it was tested the effect of fluconazole as an effective anti-*Candida* agent and of tetracycline as an anti-Lactobacilli agent, the results obtained being shown in Figure 6. As it can be

seen the two *Candida* species are not able to grow on MRS plates supplemented with 32 and 64 mg/L fluconazole (Figure 6 panel A and B), for which these plates were used to follow growth of the Lactobacilli in the co-culture system since their growth was unaffected by the presence of this drug in the medium (Figure 6 panel C and D). Similarly, the two Lactobacilli did not grow on MRS medium containing 300 mg/L tetracycline (Figure 6 panel C and D), and therefore these plates were used to follow growth of the two *Candida* species in the co-culture which were not affected by the presence of this antibiotic in the medium (Figure 6 panel A and B).

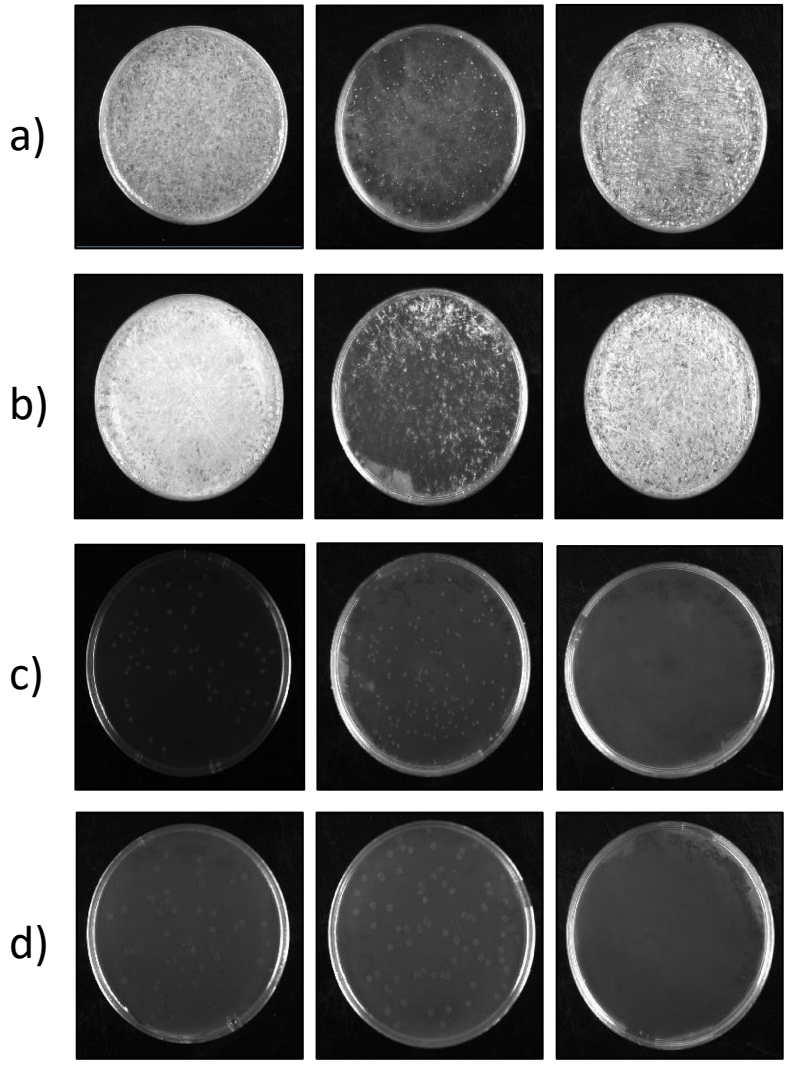

Figure 6 – a) *C. glabrata* plated in YPD, 64 mg/L fluconazole YPD and 300 mg/L tetracycline YPD, respectively; b) *C. albicans* plated in YPD, 32 mg/L fluconazole YPD and 300 mg/L tetracycline YPD, respectively; c) *L. jensenii* plated in MRS, 32 mg/L fluconazole MRS and 300 mg/L tetracycline MRS, respectively; d) *L. gasseri* plated in MRS, 32 mg/L fluconazole MRS and 300 mg/L tetracycline MRS, respectively

Co-cultures between *L. gasseri*, *L. jensenii* and *C. albicans*

C. albicans and *L. gasseri*

Using the above-defined experimental setup it was tested the effect of the presence of *L. gasseri* in growth of *C. albicans* and vice-versa. The results obtained are shown in Figure 7. Three

proportions between bacteria and yeast cells were tested: 1:1 (yeast:bacteria, both applied at the OD_{600nm} of 0.1); 1:2 (OD_{600nm} of 0.2 and 0.1 for bacteria and yeast, respectively) and 1:4 (OD_{600nm} of 0.4 and 0.1 for bacteria and yeast, respectively). The results obtained showed a decrease (of about 28%) in the growth rate of *C. albicans* when cultivated in the presence of *L. gasseri* (Table 3), while growth rate of the bacteria was inhibited by 52%, as detailed in Table 3. Necessarily, the decrease of *C. albicans* growth rate observed was more evident as the proportion of bacteria increased. After 48h there were no significant differences between viability of *C. albicans* cells cultivated in the presence or absence of *L. gasseri* (Figure 7 panel A)

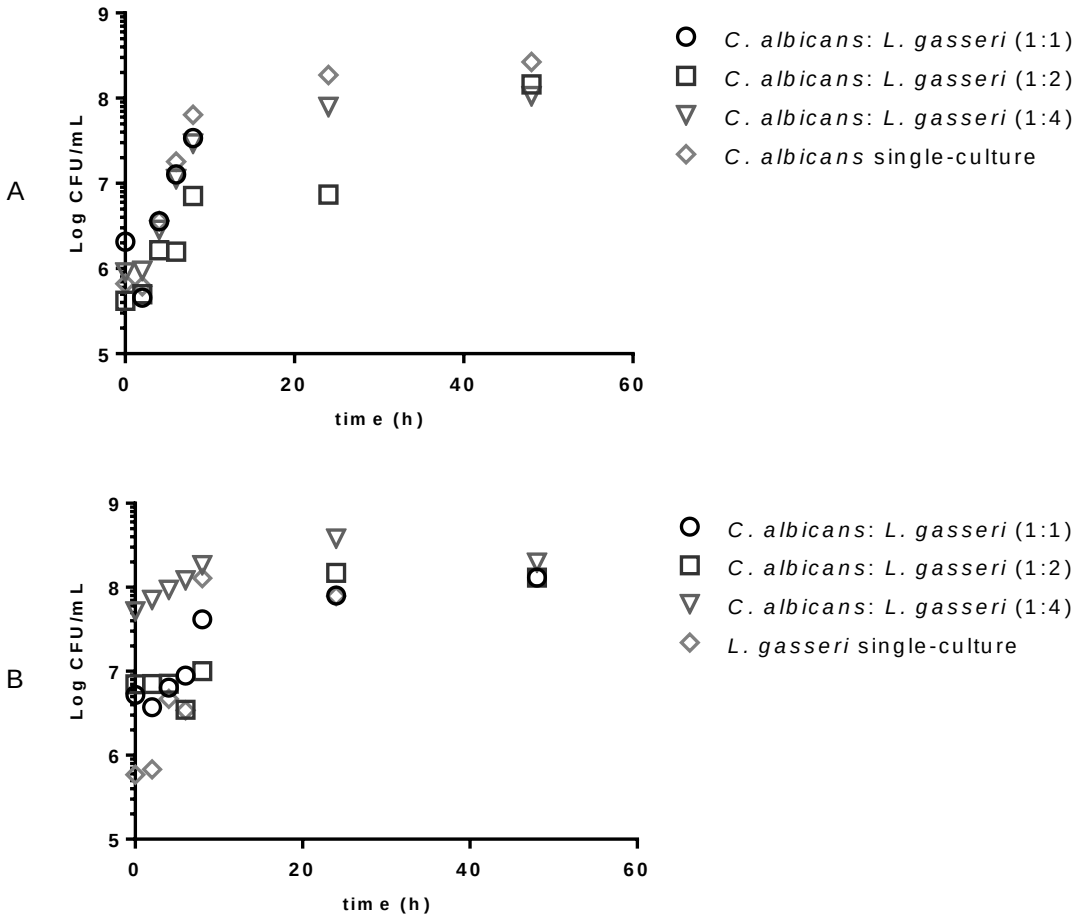

Figure 7 – A - **Growth of *C. albicans* in the presence of different concentrations of *L. gasseri*, as determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline; B - Growth of different concentrations of *L. gasseri* in the presence of *C. albicans*, as determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole**

Table 3 – Growth rates (h^{-1}), lactic acid production rate (h^{-1}), glucose production rate (h^{-1}) and final ethanol concentration (%) of *C. albicans* and *L. gasseri* in co-culture with different proportions (yeast:bacteria) at 37ºC, 100rpm and MRS medium. The results shown were obtained from two (in the case of the *L. gasseri* single-cultures) or three (in the case of *C. albicans* single-cultures and *C. albicans* + *L. gasseri* (1:4)) independent experiments

Culture	*L. gasseri* growth rate (h^{-1})	*C. albicans* growth rate (h^{-1})	Lactic acid production rate (h^{-1})	Glucose consumption rate (h^{-1})	Final ethanol concentration (%)
L. gasseri single culture	0.25±0.05	----------	0.17	0.09	0
C. albicans	----------	0.35±0.09	0.00±0.00	0.69±0.18	0.52±0.04
C. albicans + *L. gasseri* (1:1)	0.20	0.31	0.2	0.7	0.32
C. albicans + *L. gasseri* (1:2)	0.12	0.31	0.15	0.9	0.42
C. albicans + *L. gasseri* (1:4)	0.12±0.11	0.25 ±0.02	0.14±0.01	1.01±0.1	0.42±0.11

To get further insights into how growth of Lactobacilli and of *Candida* species was taking place in MRS growth medium it was also accompanied the rate of consumption of glucose and the production of lactic acid and of ethanol, two expected fermentation products. In *L. gasseri* single-culture it was observed a steep increase of lactic acid (achieving a maximum of 4.07 g/L) accompanied by a reduction in the concentration of glucose available in the broth Figure 8, panel A. Concerning *C. albicans* single-culture it was observed, as expected the consumption of glucose and the production of ethanol (6.4 %) (Figure 8 panel B and C) which confirms that under the conditions used there is limited concentration of O_2 (a requirement to improve growth of *L. gasseri*). No lactic acid was produced in *C. albicans* single culture as expected (Figure 8 panel A). It was quite noticeable the much higher rate of glucose consumption by *C. albicans* then by *L. gasseri*. The co-culture setting in the different proportion ratios of yeast:bacteria tested led to a detectable increase in the glucose consumption rate and an improvement in the final amount of lactic acid produced (Figure 8 panel A and B), only comparable to 24h to the single-culture. Both these two differences are likely to result from an improvement in the number of bacterial cells that are present in the mixed cultures.

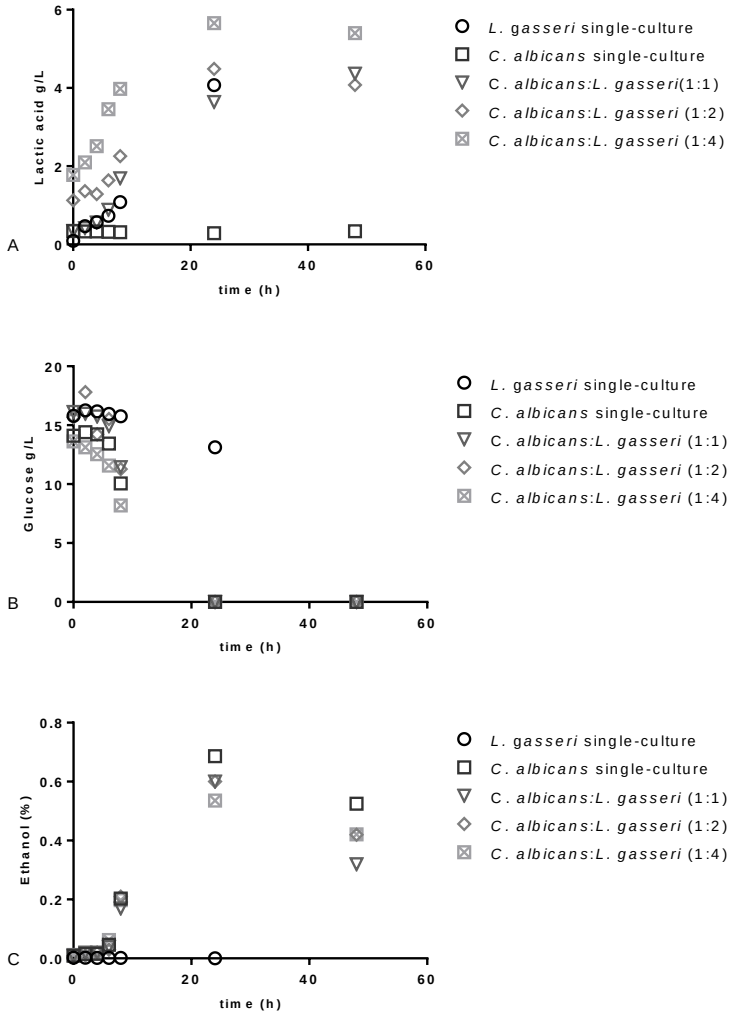

Figure 8 – Glucose (g/L), lactic acid (g/L) and ethanol (%) concentration over time (h) for *C. albicans* single-culture, *L. gasseri* single-culture and respective co-cultures

C. albicans* and *L. jensenii

Regarding the effect of *L. jensenii* on growth of *C. albicans* it was not observed any significant effect in the growth rate or viability of the yeast cells in 1:1 and 1:2 (yeast:bacteria) proportion but with the 1:4 proportion a decrease of about 34% in growth rate observed (Table 4). In the case of *L. jensenii* it was observed a decrease in the growth rate of about 57% and in cell viability of about 70% after 24h of co-cultivation.

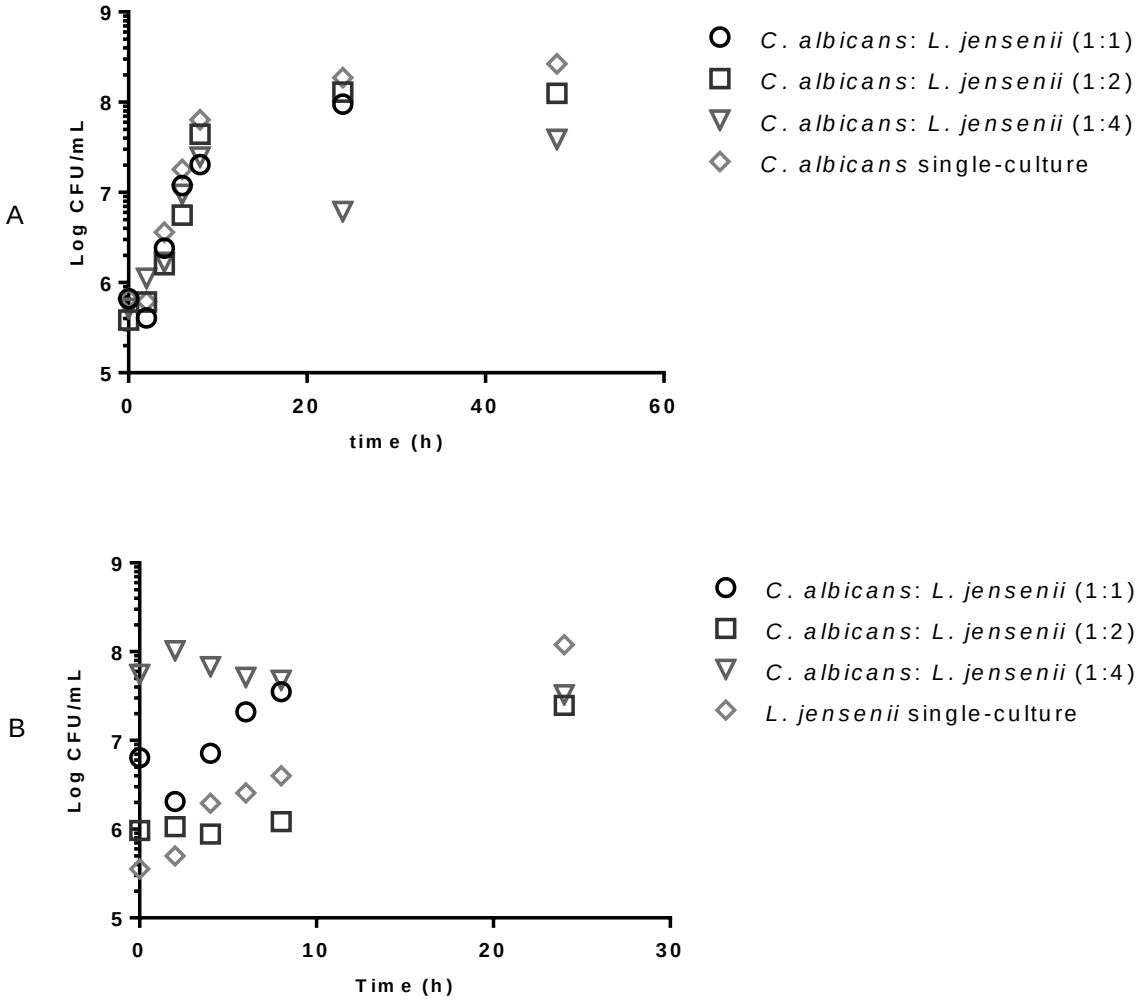

Figure 9 – A - Growth of *C. albicans* in the presence of different concentrations of *L. jensenii*, as determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline; B - Growth of different concentrations of *L. jensenii* in the presence of *C. albicans*, as determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole

Table 4 – Growth rates (h^{-1}), lactic acid production rate (h^{-1}), glucose production rate (h^{-1}) and final ethanol concentration (%) of *C. albicans* and *L. jensenii* in co-culture with different proportions (yeast:bacteria) at 37ºC, 100rpm and MRS medium. The results shown were obtained from two (in the case of the *L. jensenii* single-cultures and *C. albicans* + *L. jensenii* (1:4)) or three (in the case of *C. albicans* single-culture) independent experiments

Culture	*L. jensenii* growth rate (h^{-1})	*C. albicans* growth rate (h^{-1})	Lactic acid production rate (h^{-1})	Glucose consumption rate (h^{-1})	Final ethanol concentration (%)
L. jensenii single culture	0.17±4.38E-03	----------	0.09	0.02	0
C. albicans	----------	0.35±0.09	0.00±0.00	0.69±0.18	0.52±0.04
C. albicans + *L. jensenii* (1:1)	0.16	0.37	0.81	1.05	0.40
C. albicans + *L. jensenii* (1:2)	0.05	0.36	0.06	0.62	0.50
C. albicans + *L. jensenii* (1:4)	0.06±2.62E-03	0.23±0.02	0.26±0.02	0.5±0.3	0.47±0.12

Surprisingly, the single-culture of *L. jensenii* did not reveal a relevant consumption of glucose since after 24h it was detected about 16 g/L of the 20g/L initially provided (Figure 10 panel B). Consequently, not significant production of lactic acid was registered (Figure 10 panel A). In the co-culture setting it was observed an increase in the amount of lactic acid present in the fermentation broth, this attaining a higher level when a proportion of 1:1 was used. This result was somehow surprising since it was expected that the higher number of bacterial cells used in the 1:2 and in the 1:4 would result in enhanced production of lactic acid.

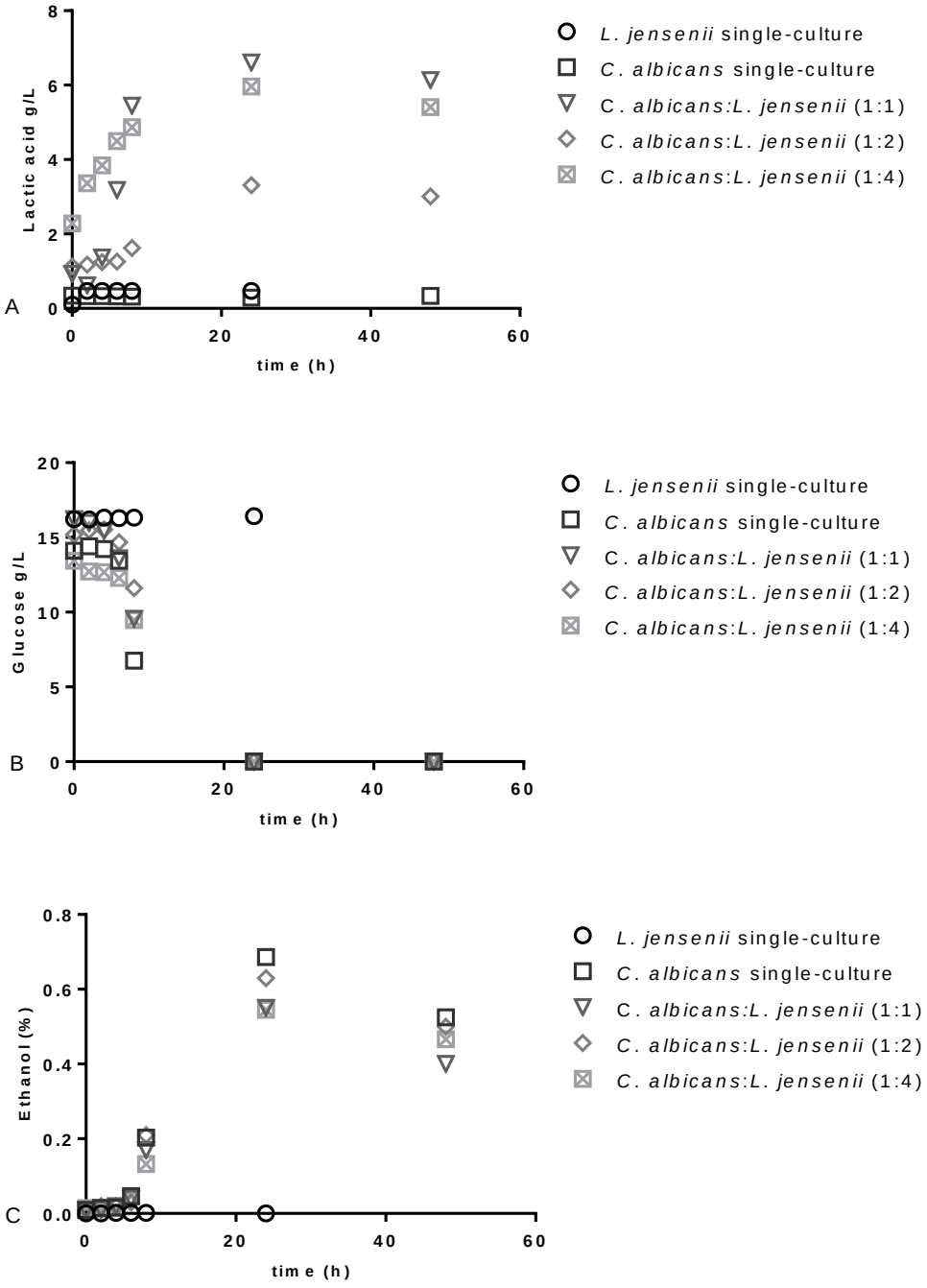

Figure 10 – Glucose (g/L), lactic acid (g/L) and ethanol (%) concentration over time (h) for *C. albicans* single-culture, *L. jensenii* single-culture and respective co-cultures

Co-culture between *L. gasseri*, *L. jensenii* and *C. glabrata*

C. glabrata and *L. gasseri*

Next it was evaluated the co-culture established between *C. glabrata* and *L. gasseri* or *L. jensenii*. The results obtained in the co-culture between *C. glabrata* and *L. gasseri* are represented in Figure 11 and Table 5. On the overall it can be concluded that under the proportions of yeast:bacteria studied both the growth rate of *L. gasseri* and *C. glabrata* was reduced (48% and 58%, respectively) in the co-culture setting (Table 5), comparing to the one obtained in single-culture (Table 5). The viability of *C. glabrata* cells was found to be reduced at the 1:4 proportion, specially in comparison with the levels attained in the single culture (Figure 11 panel A). Notably, viability of *L. gasseri* cells was observed no major fluctuations at 24h (Figure 11 panel B).

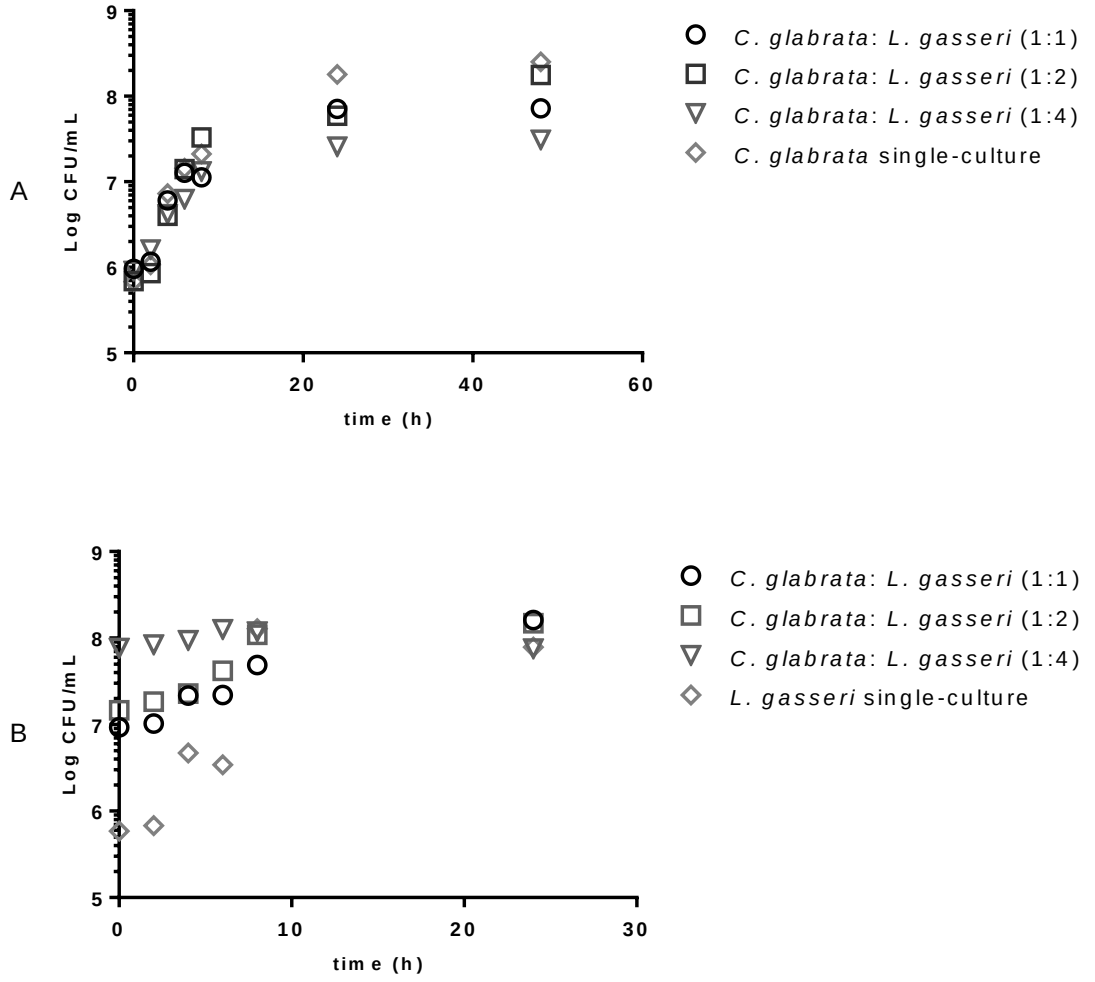

Figure 11 – A - Growth of *C. glabrata* in the presence of different concentrations of *L. gasseri*, as determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline; B - Growth of *L. gasseri* in the presence of *C. glabrata*, as determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole

Table 5 – Growth rates (h^{-1}), lactic acid production rate (h^{-1}), glucose production rate (h^{-1}) and final ethanol concentration (%) of *C. glabrata* and *L. gasseri* in co-culture with different proportions (yeast:bacteria) at 37ºC, 100rpm and MRS medium. The results shown were obtained from two (in the case of the *L. gasseri* single-cultures), three (in the case of *C. glabrata* single-culture) or four (in the case of *C. glabrata* + *L. gasseri* (1:4)) independent experiments

Culture	*L. gasseri* growth rate (h^{-1})	*C. glabrata* growth rate (h^{-1})	Lactic acid production rate (h^{-1})	Glucose consumption rate (h^{-1})	Final ethanol concentration (%)
L. gasseri single culture	0.25±0.05	----------	0.17	0.09	0
C. glabrata	----------	0.31±0.03	0.00±0.00	0.58±0.17	0.67±0.08
C. glabrata + *L. gasseri* (1:1)	0.19	0.26	0.15	0.59	0.52
C. glabrata + *L. gasseri* (1:2)	0.17	0.23	0.26	0.99	0.51
C. glabrata + *L. gasseri* (1:4)	0.13±0.03	0.13±0.05	0.25±0.11	1.08±0.15	0.45±0.04

No lactic acid was present in the supernatant of *C. glabrata* single-cultures, while the final concentration of ethanol reached 0.67% (Figure 12 panel A and C). These observations are consistent with the idea that the experimental setting led to an O2-limited environment which resulted in fermentation of the available glucose. In the presence of *L. gasseri* ethanol production was reduced by about 32% the values attained in single cultures. About 6 g/L of lactic acid were present in the mixed-culture setting having a higher initial bacterial cell density (0.4) (using the 24h time-point as a reference).

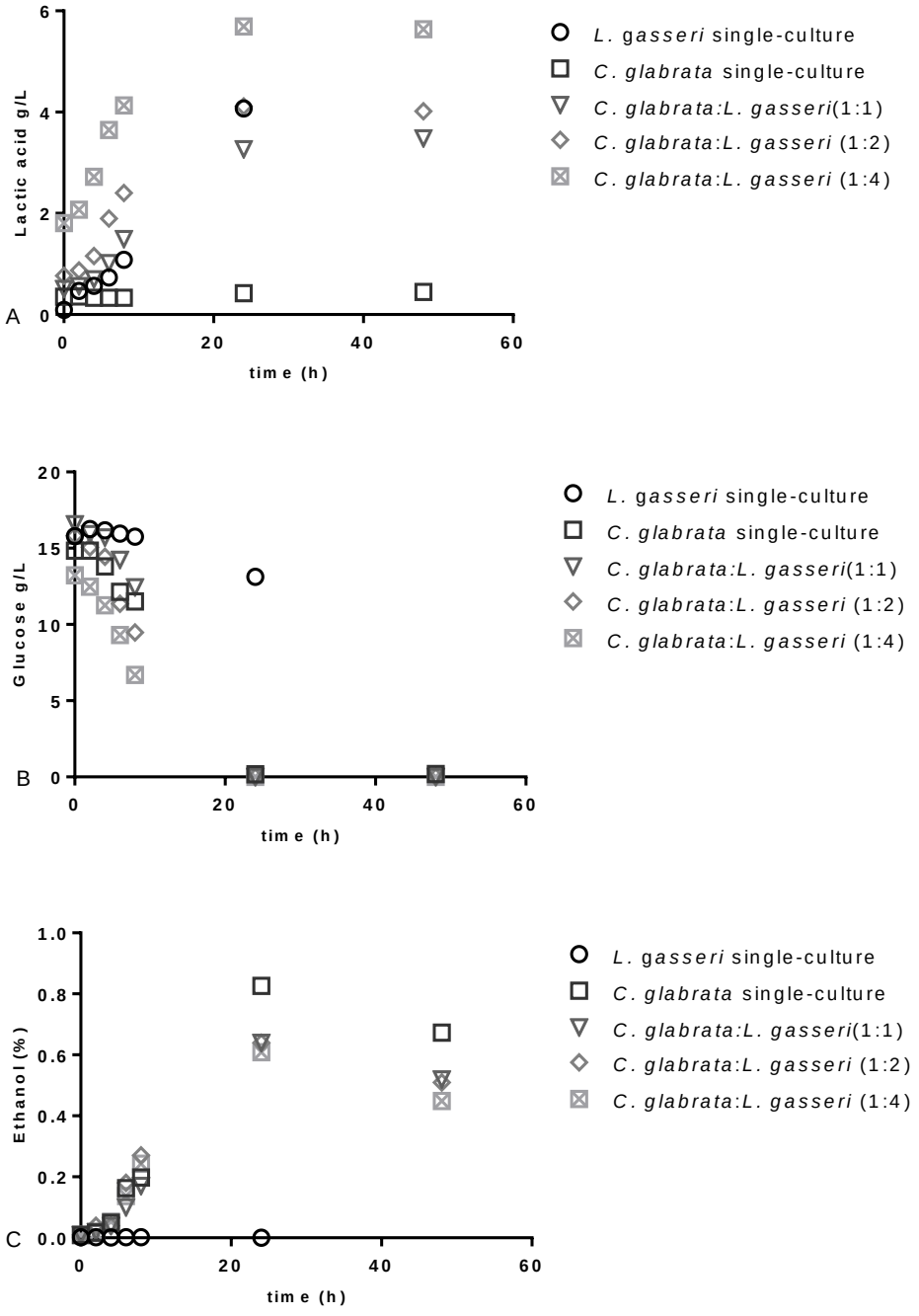

Figure 12 - Glucose (g/L), lactic acid (g/L) and ethanol (%) concentration over time (h) for *C. glabrata* single-culture, *L. gasseri* single-culture and respective co-cultures

C. glabrata* and *L. jensenii

The co-cultures with *L. jensenii* (Figure 13 and Table 6) revealed a more prominent decrease of the *C. glabrata* growth rate (approximately 54%) suggesting a higher inhibitory effect of this bacterium in inhibiting growth of this pathogenic yeast. The growth rate of *L. jensenii* (Table 6) was also reduced (about 57%) in the co-culture setting, comparing with the values registered in single-culture. A decrease in viability was also observed, specially at the higher proportion of 1:4 (Figure 13 panel B).

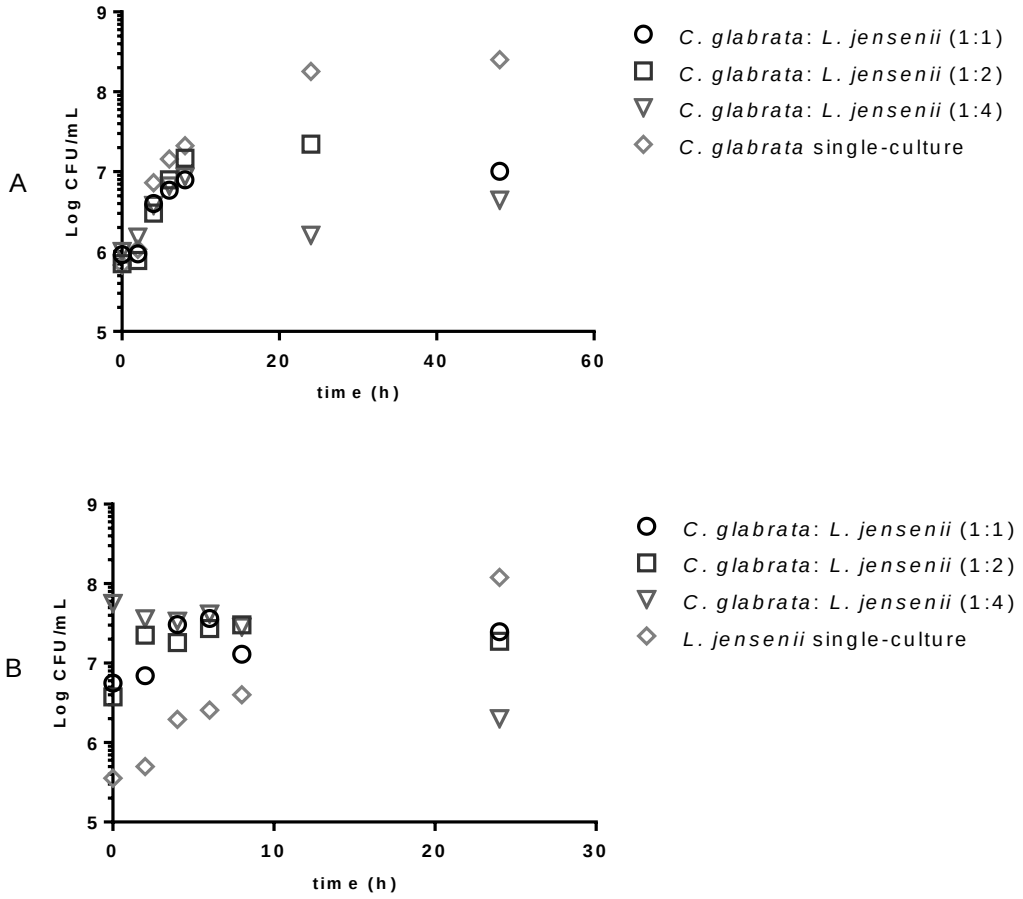

Figure 13 – A - Growth of *C. glabrata* in the presence of different concentrations of *L. jensenii*, as determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline; B - Growth of different concentrations of *L. jensenii* in the presence of *C. glabrata*, as determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole

Finally was registered the growth rate of *C. glabrata* the co-cultured with *L. jensenii*. The results are demonstrated in Table 6.

Table 6 - Growth rates (h^{-1}), lactic acid production rate (h^{-1}), glucose production rate (h^{-1}) and final ethanol concentration (%) of *C. glabrata* and *L. jensenii* in co-culture with different proportions (yeast:bacteria) of *L. jensenii* at 37ºC, 100rpm and MRS medium. The results shown were obtained from two (in the case of the *L. jensenii* single-cultures and *C. glabrata* + *L. jensenii* (1:4)) or three (in the case of *C. glabrata* single-culture) independent experiments

Culture	*L. gasseri* growth rate (h^{-1})	*C. glabrata* growth rate (h^{-1})	Lactic acid production rate (h^{-1})	Glucose consumption rate (h^{-1})	Final ethanol concentration (%)
L. jensenii single culture	0.17±4.38E-03	----------	0.09	0.02	0
C. glabrata	----------	0.31±0.03	0.00±0.00	0.58±0.17	0.67±0.08
C. glabrata + *L. jensenii* (1:1)	0.18	0.20	0.31	0.81	0.52
C. glabrata + *L. jensenii* (1:2)	0.06	0.17	0.26	0.68	0.56
C. glabrata + *L. jensenii* (1:4)	0.06±0.03	0.13±0.05	0.36±0.08	0.80±0.49	0.51±0.07

Finally, for the *C. glabrata* and *L. jensenii* co-cultures was observed decrease of about 30% of ethanol concentration in *C. glabrata* co-cultures, compared with the single-culture (Table 6) while lactic acid concentration increased in the co-culture setting (Figure 14 panel A). For glucose consumption rate, again is registered an increase with the higher bacteria proportions (Table 6**).**

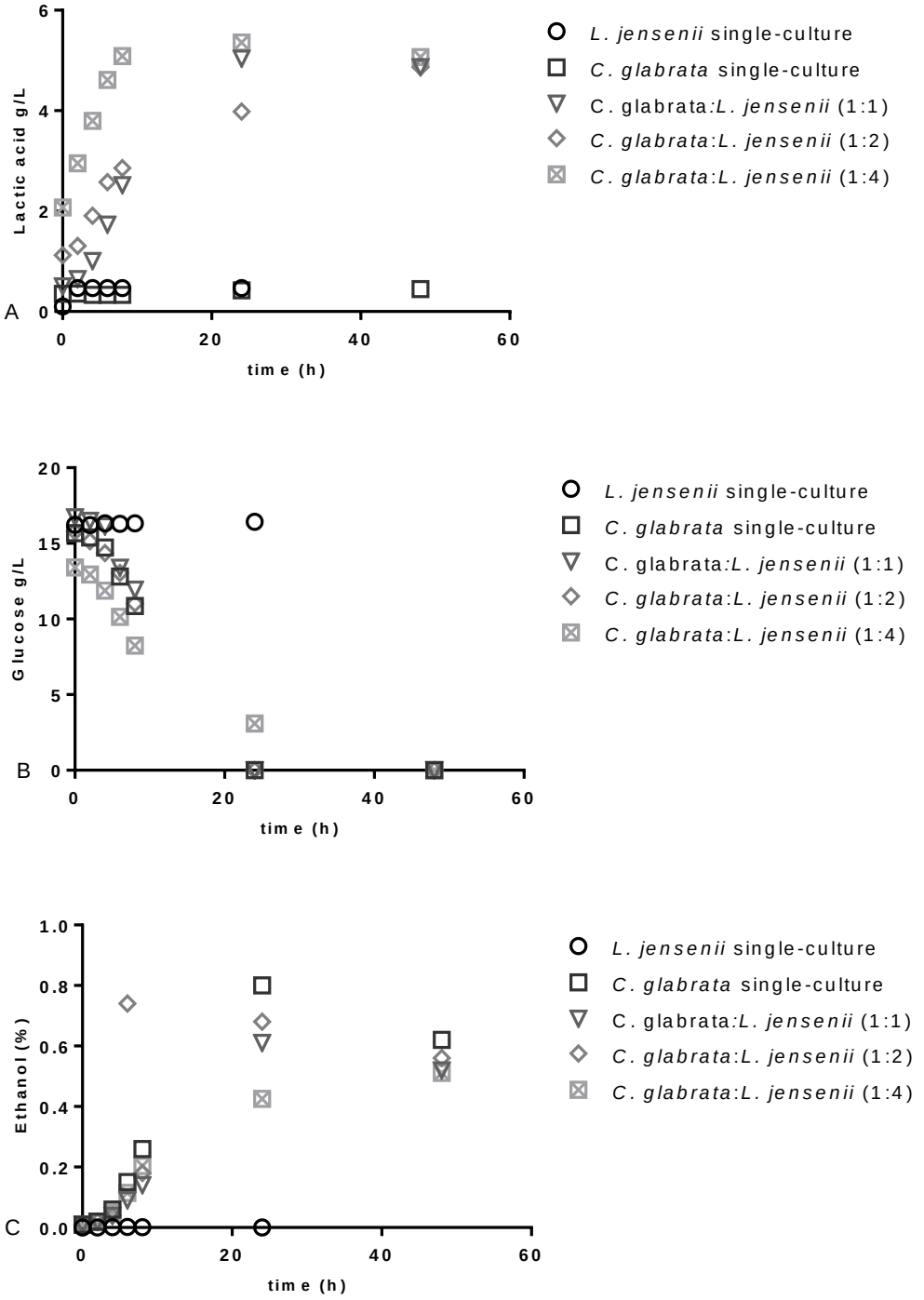

Figure 14 - Glucose (g/L), lactic acid (g/L) and ethanol (%) concentration over time (h) for *C. glabrata* single-culture, *L. jensenii* single-culture and respective co-cultures

In the next section, is performed an overall analysis of the growth parameters of both *Candida* spp, which is the major focus of this work.

Table 7 summarizes the specific growth rate for all essays performed. As we can see, both *Candida* spp showed a decrease of specific growth rate compared with the individual condition and with the increase of Lactobacilli proportion. Both *C. albicans* and *C. glabrata* alone had higher specific growth rate, showing a similar decrease in the presence of *L. gasseri* and *L. jensenii*. This reduction might be explained by the production of some molecules that inhibit *Candida spp,* for example bacteriocins, by competition for an important nutrient or by an unknown factor that is now yet documented.

Table 7 –Specific growth rate of all the tested conditions. Summary of *Candida* spp growth rate (h^{-1}), lactic acid production rate (h^{-1}), glucose production rate (h^{-1}) and final ethanol concentration (%) parameters

Culture	Yeast:bacteria cell proportion	Growth rate (h^{-1})	Lactic acid production rate (h^{-1})	Glucose consumption rate (h^{-1})	Final ethanol concentration (%)
C. albicans	**-**	**0.35±0.09**	**0.00±0.00**	**0.69±0.18**	**0.52±0.04**
C. glabrata	**-**	**0.31±0.03**	**0.00±0.00**	**0.58±0.17**	**0.67±0.08**
	1	**0.37**	**0.81**	**1.05**	**0.40**
C. albicans* and *L. jensenii	**2**	**0.36**	**0.06**	**0.62**	**0.50**
	4	**0.23±0.02**	**0.26±0.02**	**0.5±0.3**	**0.47±0.12**
	1	**0.31**	**0.2**	**0.7**	**0.32**
C. albicans* and *L. gasseri	**2**	**0.31**	**0.15**	**0.9**	**0.42**
	4	**0.25 ±0.02**	**0.14±0.01**	**1.01±0.1**	**0.42±0.11**
	1	**0.20**	**0.31**	**0.81**	**0.52**
C. glabrata* and *L. jensenii	**2**	**0.17**	**0.26**	**0.68**	**0.56**
	4	**0.13±0.05**	**0.36±0.08**	**0.80±0.49**	**0.51±0.07**
	1	**0.26**	**0.15**	**0.59**	**0.52**
C. glabrata* and *L. gasseri	**2**	**0.23**	**0.26**	**0.99**	**0.51**
	4	**0.13±0.05**	**0.25±0.11**	**1.08±0.15**	**0.45±0.04**

Effect of *CgHaa1* expression in growth of *C. glabrata* in the presence of *L. gasseri*.

As said above in the introduction section, the gene *CgHaa1* was found to play a prominent role in response of *C. glabrata* to acetic acid stress. Under the experimental conditions used, we could not detect the presence of acetic acid in the culture supernatants, but lactic acid was produced (in the range of 4-7 g/L for *C. albicans* co-cultures and 4-6 g/L for *C. glabrata* co-cultures). *CgHaa1* was also found to play a role in *C. glabrata* tolerance to lactic acid, although this was only observed for high concentrations of lactic acid (in the range of 600 mM[57], much above those found to be present in the co-culture supernatants. Either way, it was tested whether the expression of *CgHaa1* would benefit growth of *C. glabrata* in the presence of the bacteria. To do this, it was explored the co-culture system that was described above. The results obtained showed a higher reduction in cellular viability of Δ*haa1* mutant cells in a co-culture setting with *L. gasseri* (ratio of 1:4 yeast bacteria) comparing with the numbers obtained in a single-culture (Figure 15). Consistent with these observations, the amount of ethanol present in the supernatant of the *C. glabrata* ∆haa1+*L. gasseri* co-culture was considerably lower than the one registered in the supernatant of the *C. glabrata* ∆Haa1 single culture (Figure 16). On the overall this results point to a critical role of *CgHaa1* in *C. glabrata* response to *L. gasseri*, although this has to be further investigated. *L. gasseri* showed a greater decrease of its growth rate when placed in co-culture with *C. glabrata* ∆haa1 compared with the wild type.

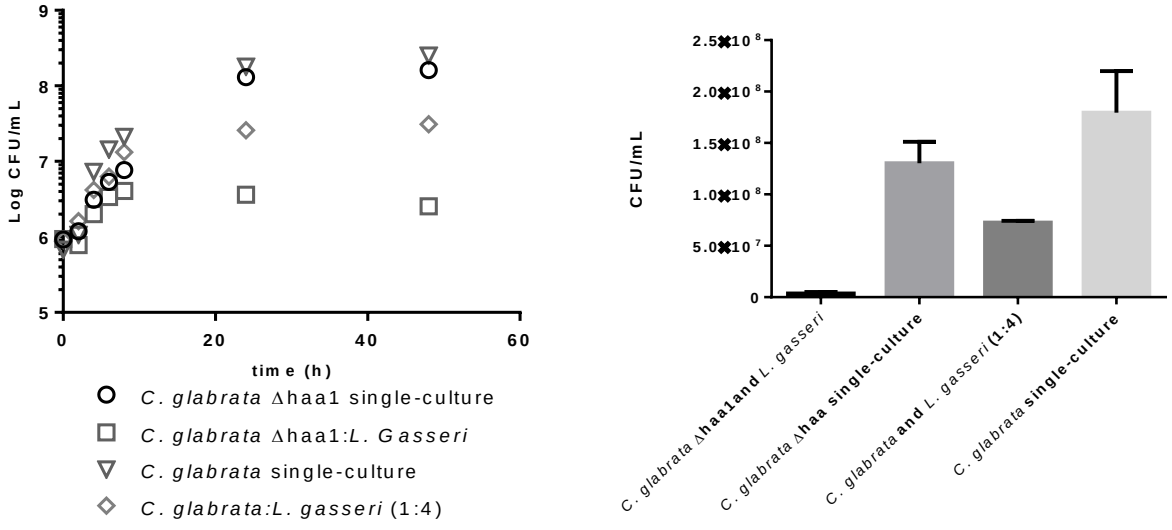

Figure 15 – Growth (left) and CFU/mL (right) of *C. glabrata* ∆haa1 and wild type in the presence of *L. gasseri*, as determined based on the number of CFUs obtained on: YPD plates supplemented with 300 mg/L tetracycline for *Candida* and MRS plates supplemented with 96 mg/L fluconazole for *L. gasseri*

Table 8 - Growth rates of *C. glabrata* Haa1 and *L. gasseri* in co-culture with 1:4 proportion (yeast:bacteria) at 37ºC, 100rpm and MRS medium. The results shown were obtained from two (in the case of the *L. gasseri* single-cultures) and three (in the case of *C. glabrata* Δhaa1 single-cultures and co-cultures) independent experiments

Culture	*L. gasseri* growth rate (h^{-1})	*C. glabrata* Δhaa1 growth rate (h^{-1})
***C. glabrata* Δhaa1 single-culture**	**----------**	**0.18±0.02**
***L. gasseri* single-culture**	**0.25±0.05**	**----------**
C. glabrata* Δhaa1 + *L. gasseri	**0.06±0.02**	**0.14±0.04**

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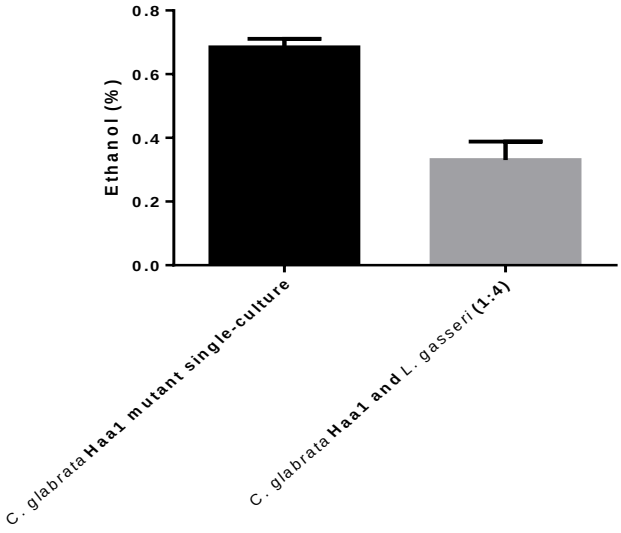

Figure 16 - Final ethanol concentration for single-culture and co-culture of *C. glabrata* Δhaa1

Effect of *L. gasseri* in genomic expression of *C. glabrata*

After having determined that the presence of *L. gasseri* inhibits growth of *C. glabrata* and showing that the expression of the transcription factor *CgHaa1* plays a detrimental role in this process it was decided to examine this at a transcriptome-wide level. For this an RNA-seq experiment was planned using the same experimental setup described above and involving the use of a 1:4 yeast to bacteria ratio. RNA-seq was chosen in order to enable assess of how the genomic expression of *C. glabrata* would respond to the presence of Lactobacilli, but also how the transcriptome of the bacterial cells changed. Due to time and economical constrains it was only possible to do the transcriptome analysis of wild-type KUE100 cells in the presence of the bacteria after 2 hours and 8 hours of inoculation, as detailed in Figure 17. These two time points were selected as being representative of an early and late phase of adaptation of the two microbes in the presence of each other.

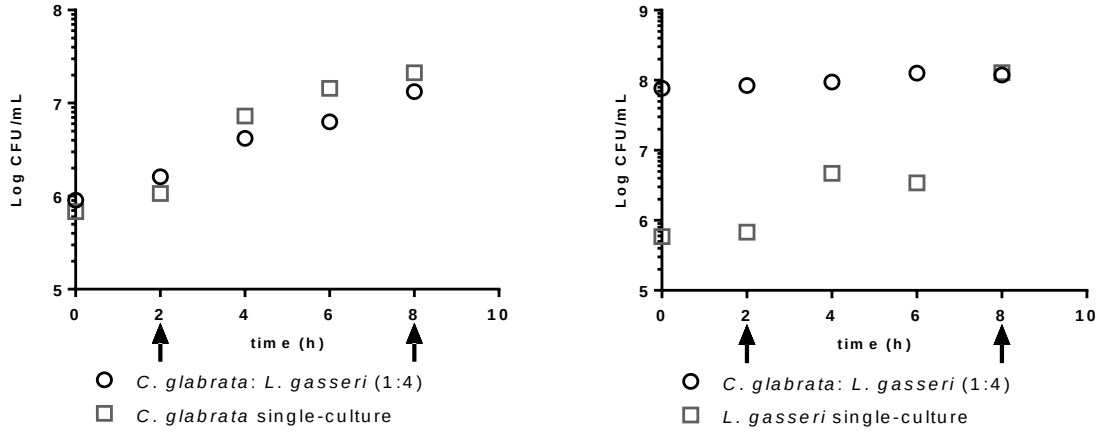

Figure 17 – Time points harvested for RNA sequencing. On the left is represented the growth curve of *C. glabrata*, and at right the growth curve of *L. gasseri*

Processing of the samples and analysis of the data obtained in the planned RNA-seq analysis was undertaken at CD Genomics (USA). This was a time-consuming task that took about 3 months to be completed and the results only arrived in our hands on mid-November. In that sense, in this thesis it was only possible to provide an overview of the results not being possible to extensively analyse the data obtained. In Table 9 are shown the results of the mapping of the reads that were obtained from the two transcriptomes against the genomes of *C. glabrata CBS138* and *L. gasseri* ATCC 33323 which were used as references.

Table 9 - Summary of the mapping results obtained for each sample

Sample ID	Total reads	rRNA rate	Reads mapped	Uniq mapped	Multi mapped
Cg (single 2h)	23592891	26.00%	22719594 (96.30%)	21915729 (92.89%)	803865 (3.41%)
Cg (single 8h)	31787714	43.13%	30762596 (96.78%)	30000220 (94.38%)	762376 (2.40%)
Cg (co-culture 2h)	32156887	26.13%	17832237 (55.45%)	17395994 (54.10%)	436243 (1.36%)
Cg (co-culture 8h)	25877569	35.07%	19606239 (75.77%)	19089115 (73.77%)	517124 (2.00%)
Lg (single 2h)	21621757	0.00%	21188429 (98.00%)	21060884 (97.41%)	127545 (0.59%)
Lg (single 8h)	25764149	0.07%	25044104 (97.21%)	24920170 (96.72%)	123934 (0.48%)
Lg (co-culture 2h)	32156887	26.13%	13497661 (41.97%)	13410945 (41.70%)	86716 (0.27%)
Lg (co-culture 8h)	25877569	35.07%	5444188 (21.04%)	5422593 (20.95%)	21595 (0.08%)

Analysis of Table 9 shows that the number of mapped reads in both single-cultures is considerably higher than the number of mapped reads in the co-cultures, which was expectable. It was also relevant to note that in the co-culture setting there was a much higher number of reads

coming from *C. glabrata* than coming from *L. gasseri* (Table 9). Significantly, the vast majority of the reads were mapped only one time in the genomes used as references, which is a highly relevant trait to assure proper estimation of gene expression based on read abundance. The expression level of a given gene was taken by counting the reads targeted in that site and taking into account the gene length as well as the sequencing depth. As such, the expression levels were measured as "FPKM" (Fragments Per Kilobase of transcript per Million mapped reads), being the most commonly used method in expression level calculation using RNA-seq data[67]. These values were then used to calculate RPKM to assess the individual gene expression. Boxplot and density plot of the RPKMs of all transcripts are used to compare the expression of different samples of *C. glabrata* and *L. gasseri* in study. From Figure 18 is observed that all samples gene expression is between 10^0 to 10^2 orders of magnitude, and very similar to each other for both *C. glabrata* genes (left) and *L. gasseri* genes (right). This result is in line with the observation that in general RPKM results span six orders of magnitude from 10^{-2} to 10^4 [68].

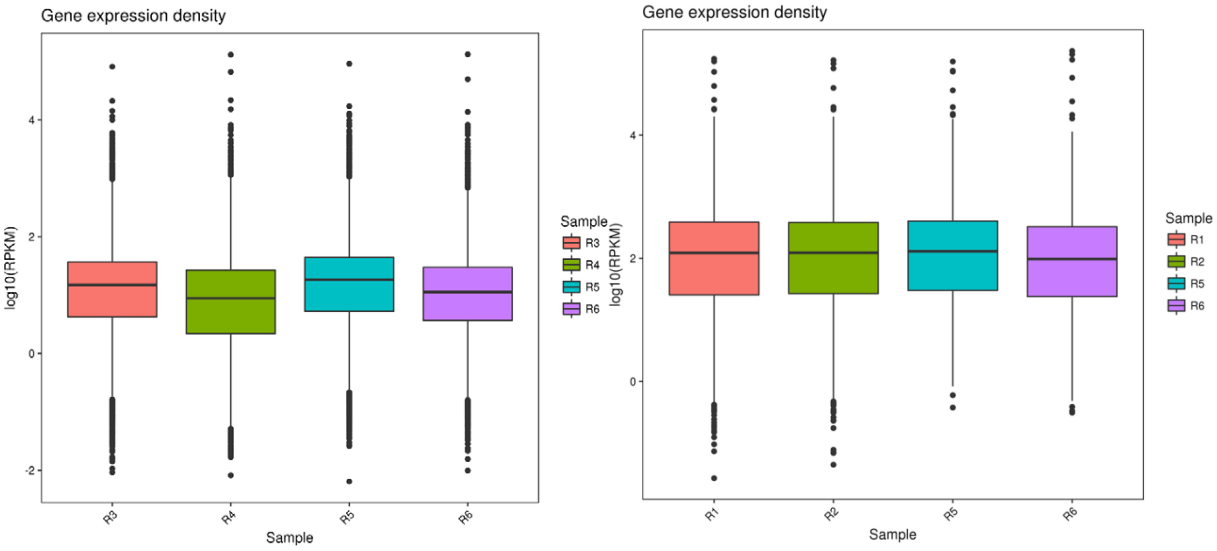

Figure 18 - Boxplot of RPKM for each sample of *C. glabrata* (left) and *L. gasseri* (right). The x-axis shows the sample names and the y-axis shows the log10(RPKM). Each box has five statistical magnitudes (max value, upper quartile, median, lower quartile and min value)

To identify the genes differently expressed in the different conditions that were examined a DIGE screening was performed using as a threshold value of 2-fold difference between conditions (co-culture vs single-culture) and an associated false discovery rate below 0.01. From this analysis it was possible to establish the set of genes differently expressed in the different conditions, these genes being shown in the volcano plots shown in Figure 19 and Figure 20. The volcano plot provides a way to perform a quick visual identification of the RNA transcripts displaying large-magnitude changes which are also statistically significant. The plot is constructed by plotting the FDR (-log10) on the y-axis, and the expression fold change (log2) between the two experimental groups on the x-axis. There are two regions of interest in the plot: those points that are found towards the top of the plot (high statistical significance) and at the extreme left or right (strongly down and up-regulated respectively).

The MA plot provides, just like the volcano plot, a quick visual identification of the RNA transcripts. With the upregulated genes on top of the graph, at red, and down regulated genes at the bottom, marked as green. The remaining volcano and MA plots are represented from the Appendix 45 to Appendix 48

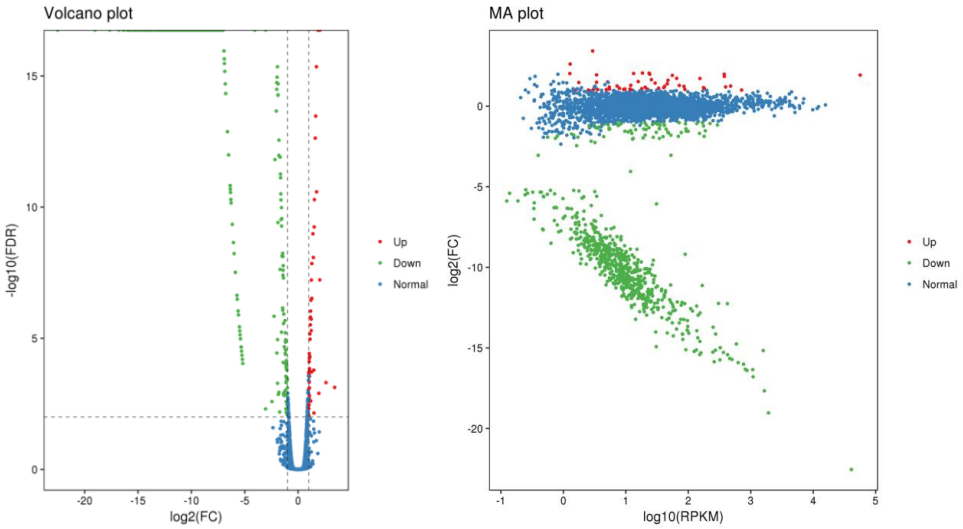

Figure 19 - Volcano plot (left) and MA plot (right) of differentially expressed genes for Cg (single 2h) vs. Cg (co-culture 2h)

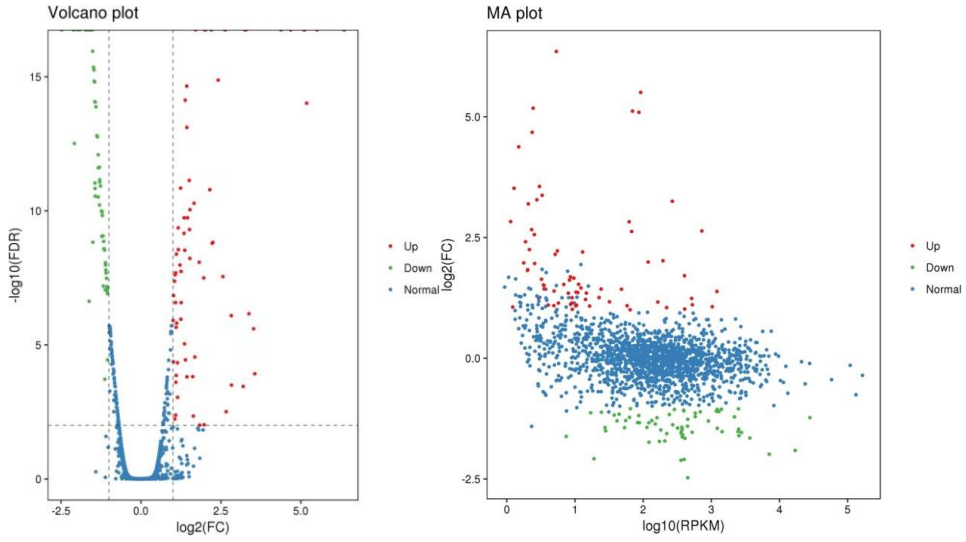

Figure 20 - Volcano plot (left) MA plot (right) of differentially expressed genes for Lg (single 2h) vs. Lg (co-culture 2h)

The information containing the entire set of genes considered being up and down-regulated genes in *C. glabrata* and *L. gasseri* in a co-culture, comparing to the levels attained in the single-culture are compiled in Table 10.

Table 10 - Number of differentially expressed genes for each pairwise comparison using as a threshold value 2-fold difference and an associated false discovery rate (FDR) below 0.01

DEG Set	All DEG	up-regulated	down-regulated
Cg (single 2h) vs Cg (co-culture 2h)	758	53	705
Cg (single 8h) vs Cg (co-culture 8h)	638	429	209
Lg (single 2h) vs Lg (co-culture 2h)	137	72	65
Lg (single 8h) vs Lg (co-culture 8h)	204	154	50

It was also constructed a Venn diagram for each specie to better visualization of the differential expressed genes of all sample comparisons (Figure 21). It was obtained a higher number of differential expressed genes for *C. glabrata*, compared with *L. gasseri*, indicating that the tested conditions leads to a more complex response of *C. glabrata* compared with *L. gasseri*.

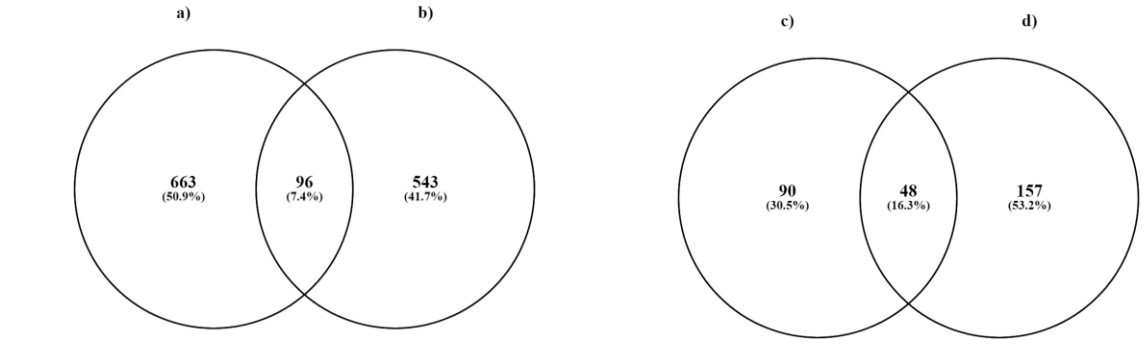

Figure 21 - Veen diagram of differential expression genes of a) Cg (single 2h) vs Cg (co-culture 2h); b) Cg (single 8h) vs Cg (co-culture 8h); and c) Lg (single 2h) vs Lg (co-culture 2h); d) Lg (single 8h) vs Lg (co-culture 8h) between comparison groups. The sum of the numbers in each large circle represents the total number of differential expressed transcripts of the comparison, and the overlapping portions of the circles represent the common differential transcripts between the comparison groups

Functional Annotation and Enrichment for Differentially Expressed Genes

The statistics for GO classification of DEGs of Cg (single 2h) vs. Cg (single 8h) and Lg (single 2h) vs. Lg (co-culture 2h) is shown in the following Figure 22. From Appendix 49 to Appendix 52 are represented the remaining sample comparisons of both *C. glabrata* and *L. gasseri*. For better visualization, the most enriched GO terms for the same comparisons were plotted and are represented in the following Figure 23 for *C. glabrata* and Figure 24 *L. gasseri*. The rest bar plots of the most enriched go terms are represented from Appendix 53 to Appendix 56.

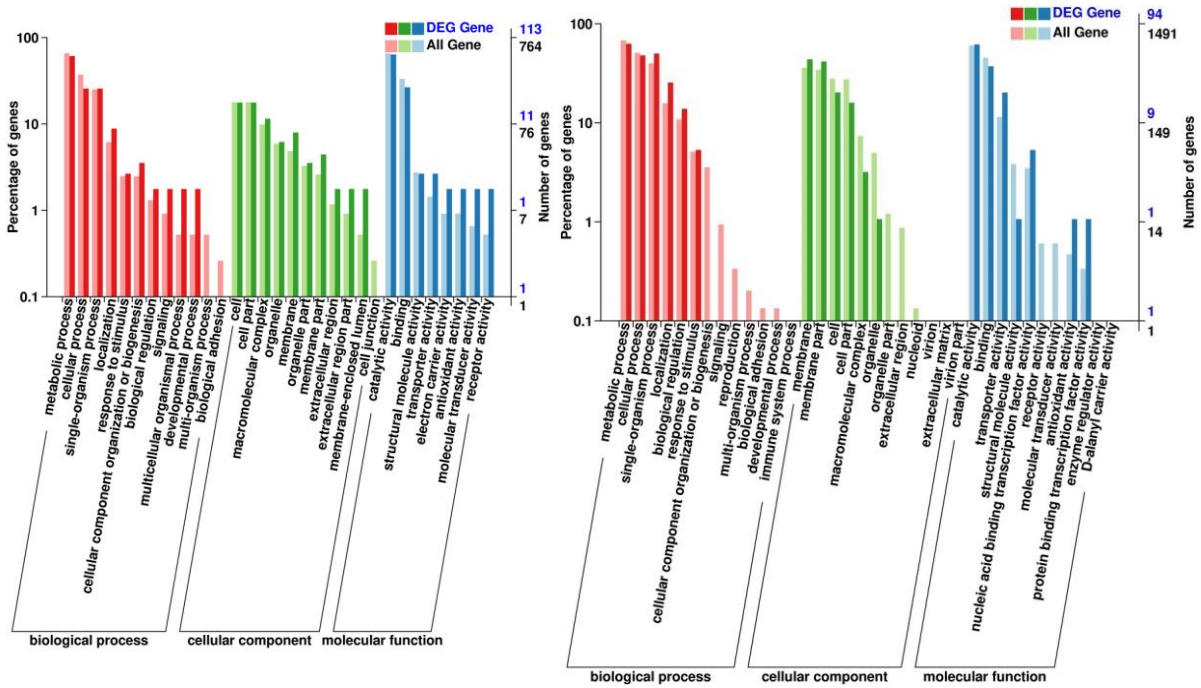

Figure 22 - Statistics results of GO annotation for Cg (single 2h) vs. Cg (co-culture 2h) DEGs (left) and Lg (single 2h) vs. Lg (co-culture 2h) DEGs (right)

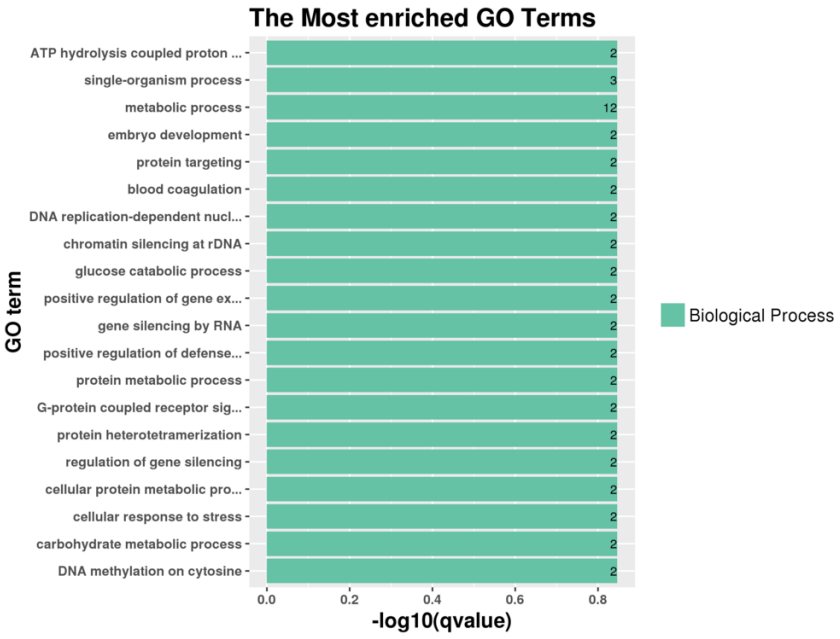

Figure 23 - Bar plot of enriched GO term of *C. glabrata* differentially expressed genes (up and down regulated) for Cg (single 2h) vs. Cg (co-culture 2h). The ordinate is the enriched GO term, the abscissa is the q-value of the term enrichment, and the number of differentially expressed genes is shown on the column. Different colors are used to distinguish biological process, cellular component and molecular function

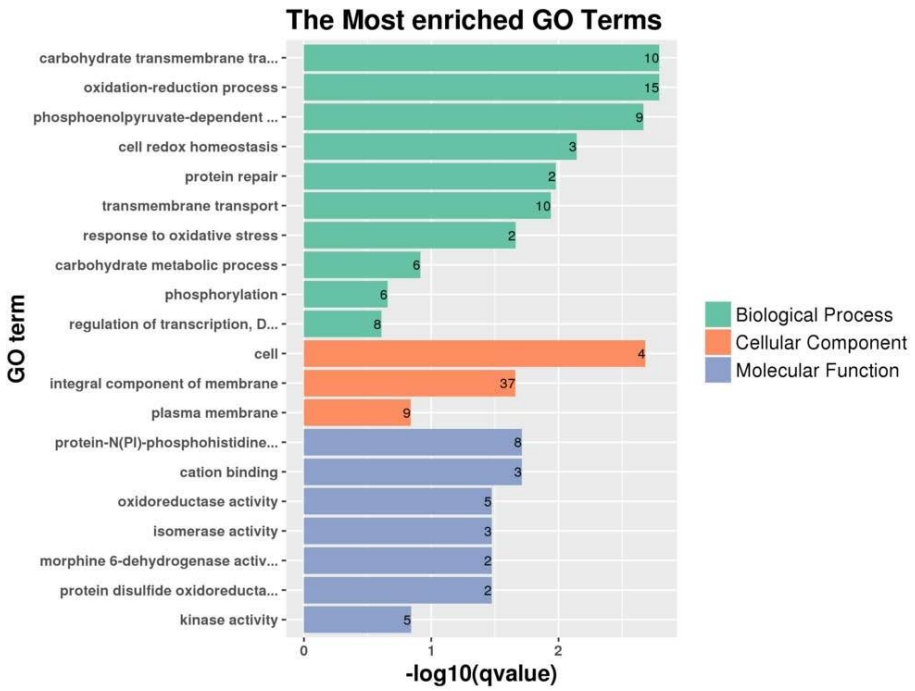

Figure 24 - Bar plot of enriched GO term of *L. gasseri* differentially expressed (up and down regulated) genes for Lg (single 2h) vs. Lg (co-culture 2h). The ordinate is the enriched GO term, the abscissa is the q-value of the term enrichment, and the number of differentially expressed genes is shown on the column. Different colours are used to distinguish biological process, cellular component and molecular function

From this analysis it was possible to observe that the higher number of *C. glabrata* genes found to be differently expressed (either up or down-regulated) in the co-culture setting are mostly involved in metabolic functions including for example magnesium-activated aldehyde dehydrogenase or D-3-phosphoglycerate dehydrogenases. Regarding *L. gasseri* DEGs the more common GO terms affected were carbohydrate transmembrane transport, oxidation-reduction process and transmembrane transport, for example. Further studies are now required to properly interpret these results, namely to better distinguish the functional clustering's relevant within the genes that were found to be specifically up- or down-regulated in the co-culture setting.

Effect of adaptation to the vaginal environment in the ability of *C. glabrata* and *C. albicans* to grow in the presence of *L. gasseri*

Adaptation of *C. glabrata* and *C. albicans* cells to the vaginal environment, which contains *L. gasseri*, is expected to result in the development of efficient adaptive responses that allows the yeast cells to better cope with the presence of the bacteria in the environment. In this sense, it was compared growth of a set of vaginal *C. glabrata* and *C. albicans* isolates in the presence of *L. gasseri* using the same experimental setup that has been used throughout this work. The set of *C. glabrata* isolates used had been previously characterized in a previous study[54] and also the BG2 strain, a vaginal strain that had been studied before[54,69,70] while the set of *C. albicans* used has not been characterized previously. Concerning the *C. albicans* isolates, it was visible the effect in reduction of the growth rate of the yeast cells when cultivated in the presence of *L. gasseri* (Figure 25 and Table

11). The extent of this reduction was similar (of about 30%) among the different isolates tested with the exception of Vg485 that showed a higher decrease of growth rate (48%) comparing to the one observed in the laboratory strain SC5314 (28%). Prominent decreases (ranging from 52 to 74%) in the viability of the cultures after 24h of incubation in the presence of *L. gasseri* were also observed, these being values that surpassed the viability loss that had been observed for the laboratory strain SC5314 (Figure 25 panel C).

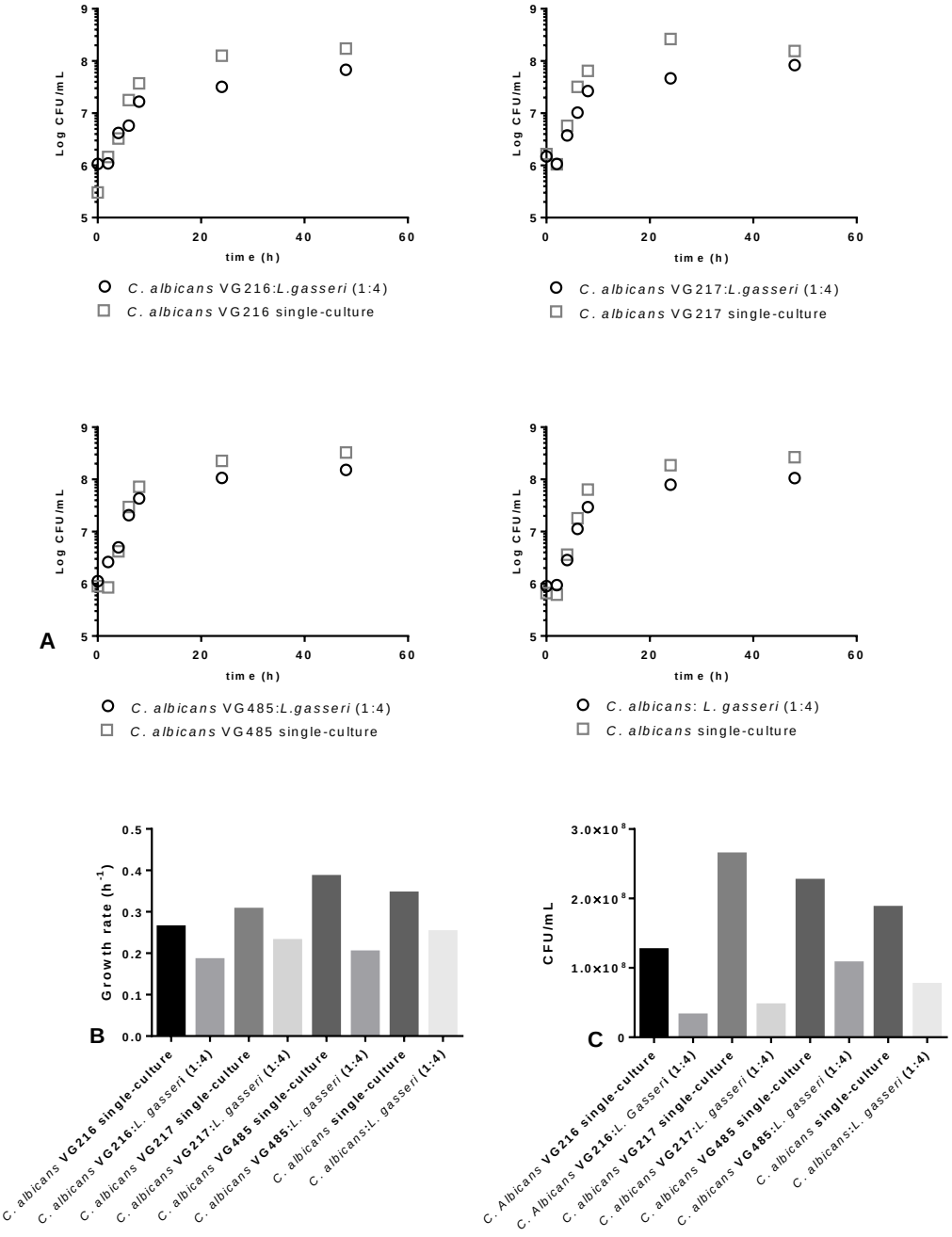

Figure 25 – A) Growth curve; B) growth rate (h^{-1}) and C) cellular viability (CFU/mL) of *C. albicans* VG216, VG217, VG485 and wild type in single-culture and in the presence of *L. gasseri*, at 37ºC, 100rpm and MRS medium

Table 11 - Growth rates of *C. albicans* vaginal isolates and *L. gasseri* in co-culture with 1:4 proportion (yeast:bacteria) at 37ºC, 100rpm and MRS medium. The results shown were obtained from two (in the case of the *L. gasseri* single-cultures) and three (in the case of *C. albicans* single-culture and *C. albicans* + *L. gasseri* (1:4)) independent experiments

Culture	*L. gasseri* growth rate (h^{-1})	*Candida* spp. growth rate (h^{-1})
***L. gasseri* single-culture**	**0.25±0.05**	**----------**
***C. albicans* single-culture**	**----------**	**0.35±0.09**
***C. albicans* + *L. gasseri* (1:4)**	**0.12±0.11**	**0.25±0.02**
***C. albicans* VG216 single-culture**	**----------**	**0.26**
***C. albicans* VG216 + *L. gasseri* (1:4)**	**0.09**	**0.18**
***C. albicans* VG217 single-culture**	**----------**	**0.31**
***C. albicans* VG217 + *L. gasseri* (1:4)**	**0.10**	**0.23**
***C. albicans* VG485 single-culture**	**----------**	**0.39**
***C. albicans* VG485 + *L. gasseri* (1:4)**	**0.08**	**0.20**

In the case of the tested *C. glabrata* isolates it was observed that the presence of *L. gasseri* decreased the growth rate (in the range of 3-12%) and also the viability of the cells after 48h in the co-culture setting (in the range of 3-70%) (Figure 26 panel B). It was particularly interesting to observe marked differences in the cell viability of the isolates, with isolate VG281 exhibiting only a minor reduction of viability in the co-culture while isolate VG216 appeared to be much more markedly susceptible to the presence of *L. gasseri* as its viability was reduced by 70% (Figure 26 panel C). Another observation of remark was the fact that the reduction of the growth rate, as well as of the cell viability, was much more pronounced in the lab strain KUE100 than in these vaginal isolates. *L. gasseri* showed for all co-culture assays with vaginal strains a decrease of growth rate of about 60% to 76%, a higher decrease when compared with co-cultured with the wild type (of about 48%). On the overall these results suggest that *C. glabrata* vaginal isolates are much better adapted to the presence of *L. gasseri*, than the laboratory strain. It was also interesting to observe that the effect of adaptation was clearly visible in the *C. glabrata* isolates, but not in the *C. albicans* ones. Further studies are required to better assess this, eventually using other cohorts of isolates.

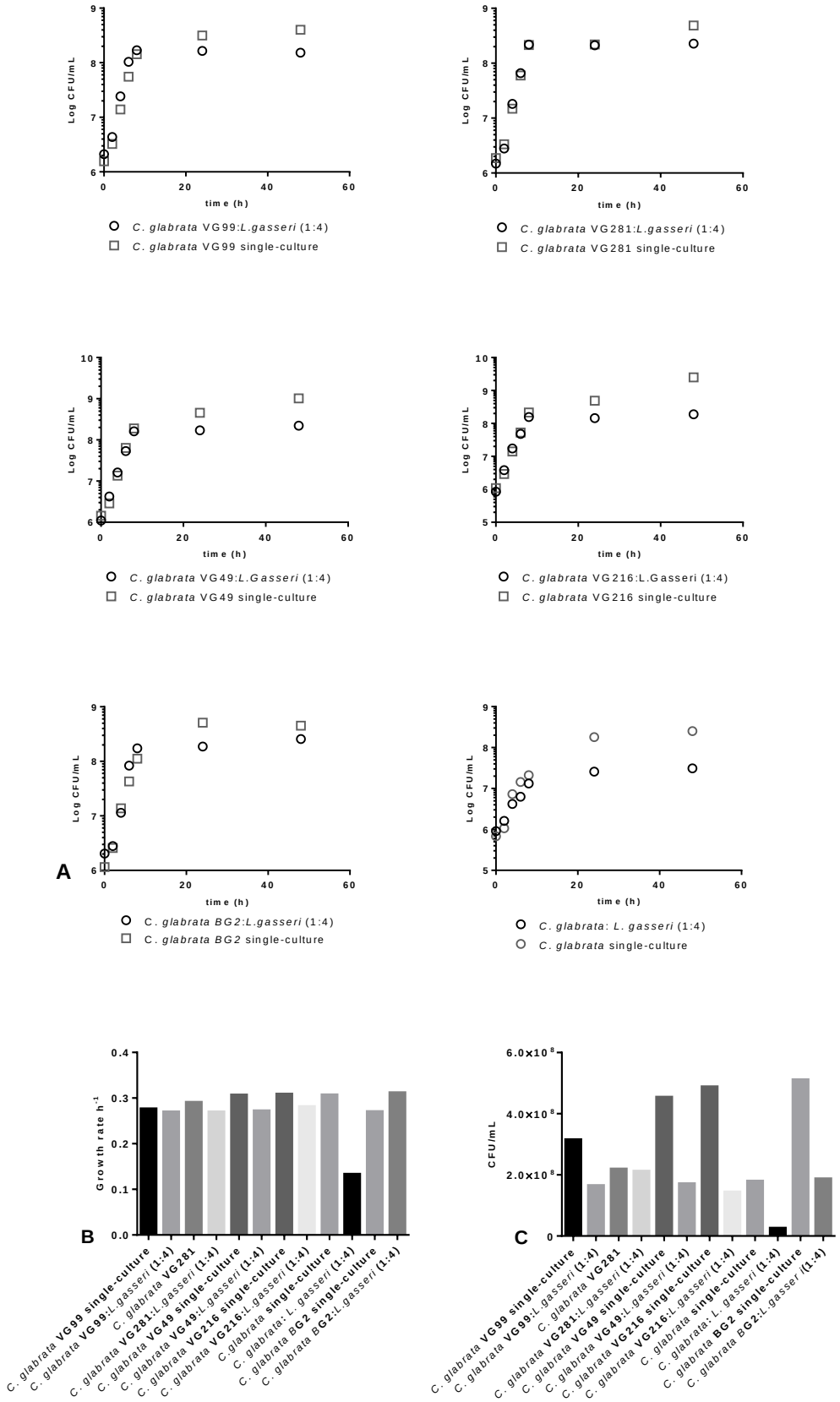

Figure 26 – A) Growth curve, B) growth rate (h^{-1}) and C) cellular viability of *C. glabrata* VG99, VG281, VG49 VG 216, BG2 and wild type in single-culture and in the presence of *L. gasseri*, at 37ºC, 100rpm and MRS medium

Table 12 - Growth rates of *C. glabrata* vaginal isolates, *C. glabrata* BG2 and *L. gasseri* in co-culture with 1:4 proportion (yeast:bacteria) at 37ºC, 100rpm and MRS medium. The results shown were obtained from two (in the case of the *L. gasseri* single-cultures), three (in the case of *C. glabrata* single-culture) and four (in the case of *C. glabrata* + *L. gasseri* (1:4)) independent experiments

Culture	*L. gasseri* growth rate (h^{-1})	*Candida* spp. growth rate (h^{-1})
L. gasseri	**0.25±0.05**	**----------**
***C. glabrata* single-culture**	**----------**	**0.31±0.03**
***C. glabrata* + *L. gasseri* (1:4)**	**0.13±0.03**	**0.13±0.05**
***C. glabrata* VG99 single-culture**	**----------**	**0.28**
***L. gasseri* + *C. glabrata* VG99 (1:4)**	**0.10**	**0.27**
***C. glabrata* VG281 single-culture**	**----------**	**0.29**
***L. gasseri* + *C. glabrata* VG281 (1:4)**	**0.10**	**0.27**
***C. glabrata* VG49 single-culture**	**----------**	**0.31**
***L. gasseri* + *C. glabrata* VG49 (1:4)**	**0.06**	**0.27**
***C. glabrata* VG216 single-culture**	**----------**	**0.31**
***L. gasseri* + *C. glabrata* VG216 (1:4)**	**0.10**	**0.28**
***C. glabrata* BG2 single-culture**	**----------**	**0.27**
***C. glabrata* BG2 + *L. gasseri* (1:4)**	**0.09**	**0.31**

HPLC analysis of the supernatants of co-cultures established between the vaginal isolates and *L. gasseri* was also performed, these results being summarized in Table 12. The patterns of lactic acid production, ethanol production, and glucose consumption observed in all assays with both *C. glabrata* and *C. albicans* vaginal isolates are similar to those observed for the laboratory strains (Appendix 1 to Appendix 21).

Effect of *L. gasseri* supernatant and live cells in growth, biofilm formation and filamentation of *C. albicans* and/or *C. glabrata*

The assays described so far have explored co-cultures of Lactobacilli with *C. albicans* or *C. glabrata*, in which the yeasts and bacteria are co-inoculated together in a fresh medium. This differs a bit from the experimental setting that has been explored in most studies demonstrating anti-candicidal effect of Lactobacilli against *Candida*[15,33,46,71], in which growth of the *Candida* spp is examined upon exposure to a supernatant obtained from a pre-culture of Lactobacilli (rather from the direct contact with the bacteria which is the approach herein explored). To have an idea on this, the susceptibility of *C. albicans* and of *C. glabrata* to a supernatant where *L. gasseri* had been cultivated for 24h was compared in 96-microwell plates. As a control the *C. albicans* or *C. glabrata* cells were exposed to fresh MRS medium. A gradient of supernatant (5,15,25,30,40,50,75 and 100 μL in a total volume of 200 μL) was used. The results obtained showed that inhibition of *C. glabrata* growth is only observed when 100 μL of the bacterial supernatant were used, being observed an increased lag phase before exponential growth is resumed (Figure 27). In the case of *C. albicans* inhibition is already observed when only 50 μL of the supernatant are used (Figure 27). It is important to stress that when only 100μL

of MRS medium are used, there was no inhibition of *C. albicans* or *C. glabrata* growth, sustaining the idea that the inhibition comes from the presence of the bacteria in medium (Figure 28).

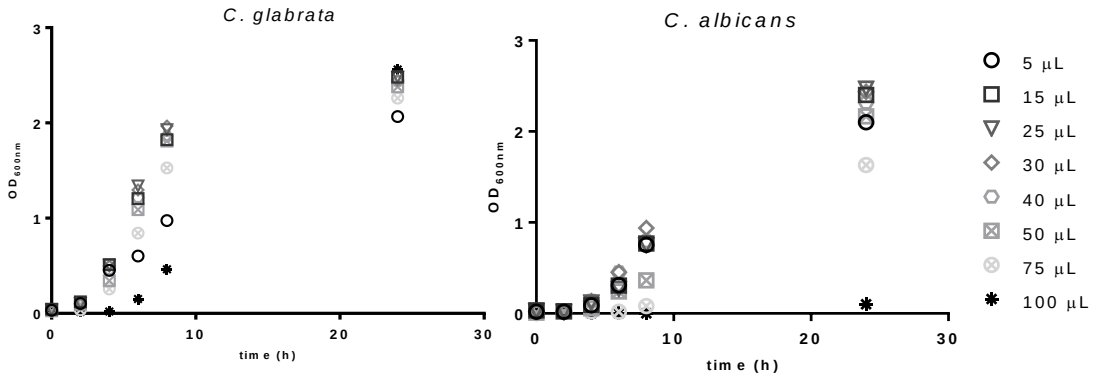

Figure 27 - Growth curve of *C. glabrata* and *C. albicans* with different concentrations of *L. gasseri* supernatant at 37ºC, 25 rpm in MMB medium

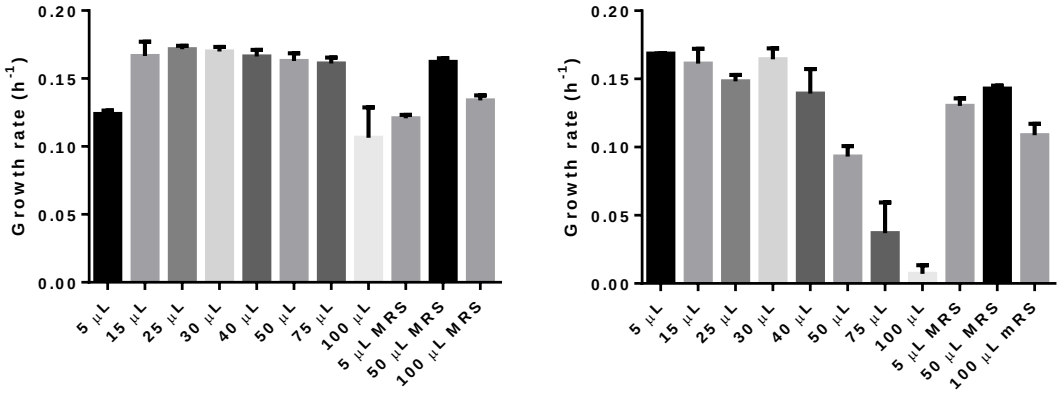

Figure 28 - Growth rate of *C. glabrata* (left) and *C. albicans* (right) with different concentrations of *L. gasseri* supernatant and MRS medium at 37ºC, 25 rpm in MMB medium. The results shown were obtained from three independent experiments

Because biofilms are such a relevant virulence trait for *C. albicans* and *C. glabrata* cells[9,13,44,72], in this work it was also examined the effect of the bacterial supernatant in the ability of these yeast species to form biofilms. As such the same experimental setting used to cultivate *C. albicans* or *C. glabrata* in the presence of the bacterial supernatant was used, with the difference that this time after 8h and 24h PrestoBlue was added to measure the amount of biofilm formed. In this case the growth medium used to cultivate *C. albicans* or *C. glabrata* was RPMI since in MM growth medium there was no significant formation of biofilm. The results obtained are compiled in Figure 29. The bacterial supernatant clearly reduced the ability of *C. albicans* or *C. glabrata* to form biofilms on the surface of the 96-multiwell polystyrene plates, nevertheless, it is important to stress that a marked reduction was also observed when only MRS medium was used Figure 29. Nevertheless, the reduction induced by the supernatant was always more evident than the one attributable to the MRS medium (Figure 29).

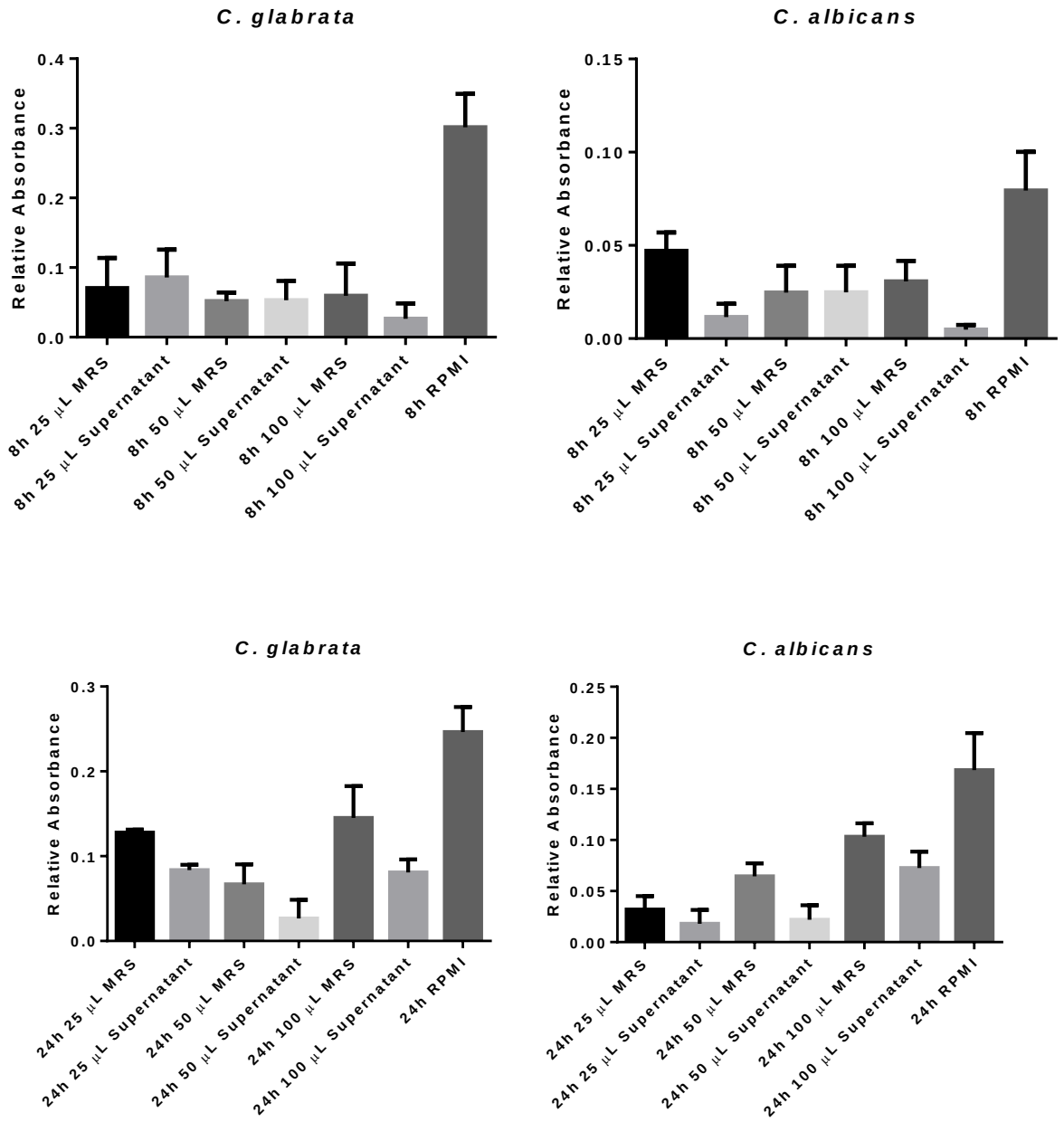

Figure 29 - Biofilm formation of *C. glabrata* and *C. albicans* at 8h and 24h in the presence of different concentration of *L. gasseri* supernatant and MRS medium at 37ºC, 25 rpm in RPMI medium. The results shown were obtained from three independent experiments

The fact that the bacterial supernatant appear to have induced a reduction in the ability of the *C. glabrata* or *C. albicans* cells to adhere to the surface of the polystyrene plates suggested that live cells could also play a similar effect. As such, a co-culture was established between *C. albicans* and *L. gasseri* or *L. jensenii* for 24h after which the cells were observed by scanning electron microscopy, this being performed in collaboration with Dr Marta Alves from CQE. In the single cultures it was possible to observe Lactobacilli as rod shaped cells that form long filaments of cells (Figure 30 and Figure 31), while *C. albicans* appeared as round shaped cells (Figure 32). Biofilm formation was observed for all the species tested. Co-cultivation resulted in the formation of a mixed biofilm comprising both *C. albicans* and Lactobacilli cells. From Figure 33 to Figure 36 (and Appendix 39 to

Appendix 44) it is possible to clearly observe the interaction between *L. gasseri* and *L .jensenii* and *C. albicans* cells. For example in Figure 34 in the 10000x magnification is easily perceived that *C. albicans* cells are surrounded by *L. jensenii* cells. In all cases the increase of *Lactobacillus* species led to an increase of cell number, showing a much more packed biofilm.

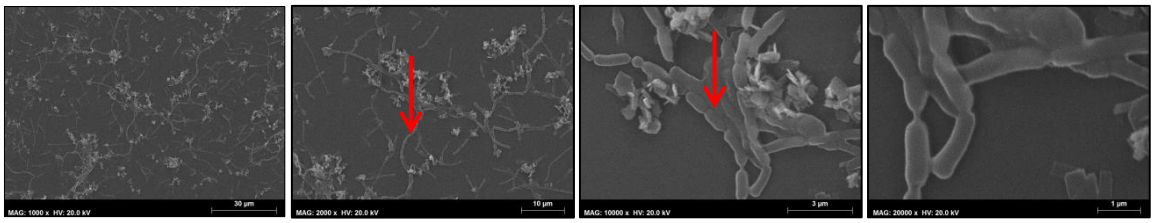

Figure 30 – *L. jensenii* cells from 0.1 initial OD culture observed by SEM with a magnification of 1000x, 2000x, 10000x and 20000x, respectively. Red arrows – *L. jensenii* cells forming filaments

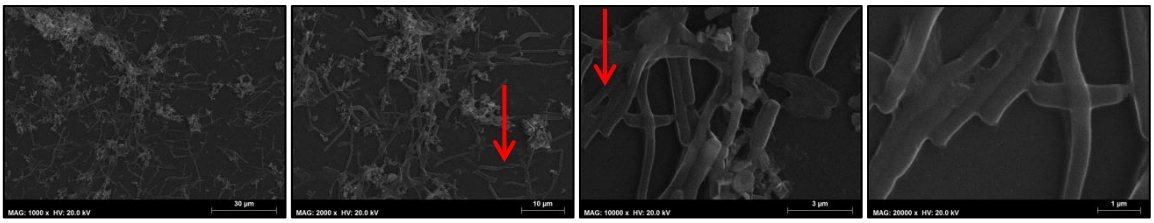

Figure 31 – *L. gasseri* cells from 0.1 initial OD culture observed by SEM with a magnification of 1000x, 2000x, 10000x and 20000x, respectively. Red arrows – *L. gasseri* cells forming filaments

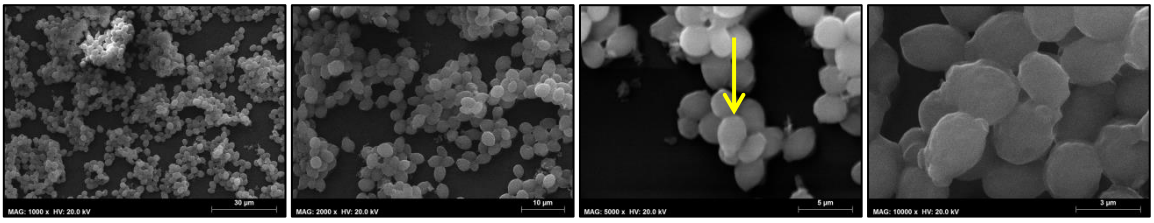

Figure 32 – *C. albicans SC 138* cells from 0.1 initial OD culture observed by SEM with a magnification of 1000x, 2000x, 10000x and 20000x, respectively. Yellow arrows – *C. albicans* cells

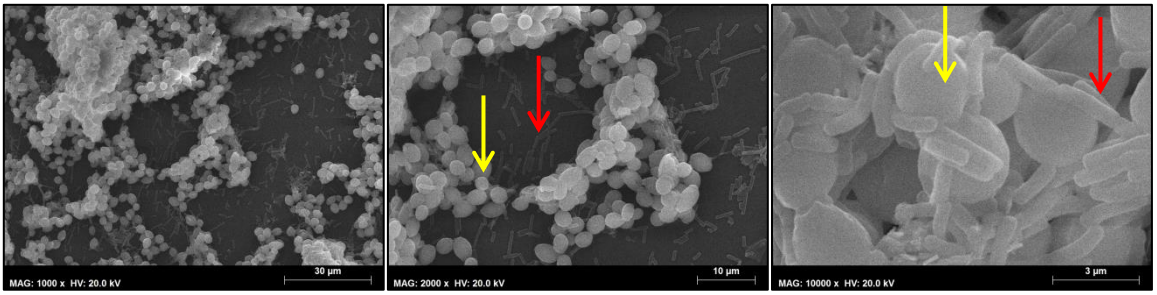

Figure 33 – Cells from *C. albicans* and *L. gasseri* from 0.1 initial OD culture observed by SEM with a magnification of 1000x, 2000x and 10000x, respectively. Yellow arrows – *C. albicans* cells; Red arrows – *L. gasseri* cells

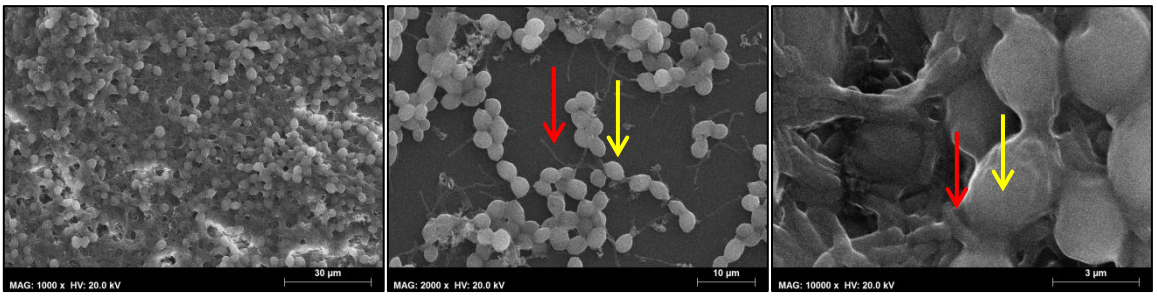

Figure 34 - Cells from *C. albicans* and *L. jensenii* from 0.1 initial OD culture observed by SEM with a magnification of 1000x, 2000x and 10000x, respectively. Yellow arrows – *C. albicans* cells; Red arrows – *L. jensenii* cells

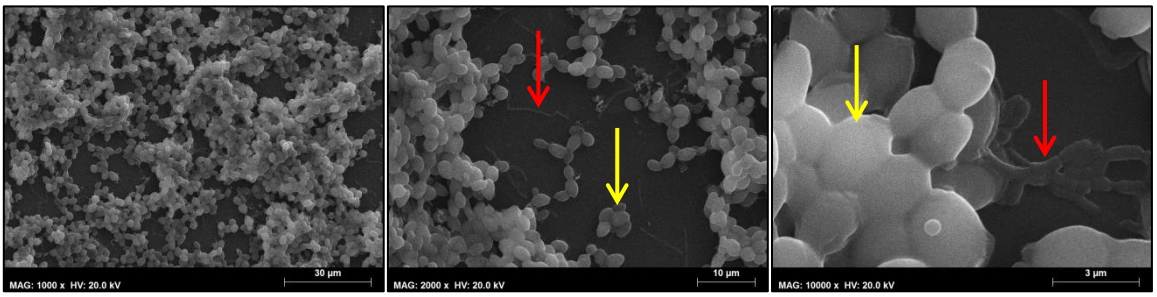

Figure 35 – Cells from *C. glabrata* and *L. jensenii* from 0.1 initial OD culture observed by SEM with a magnification of 1000x, 2000x and 10000x, respectively. Yellow arrows – *C. glabrata* cells; Red arrows – *L. jensenii* cells

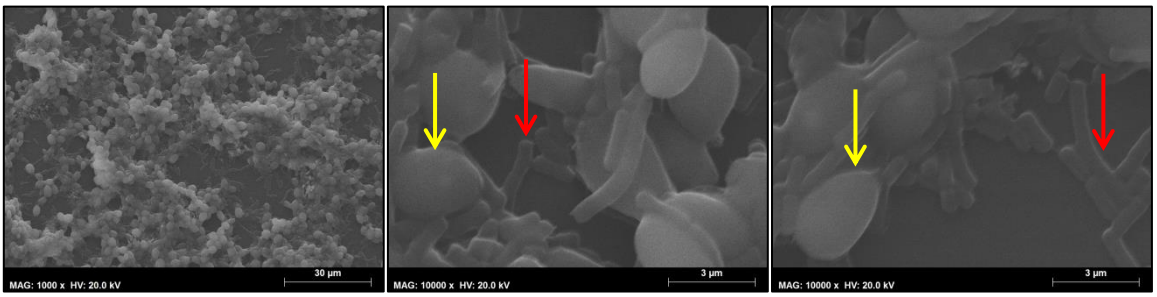

Figure 36 – Cells from *C. glabrata* and *L. gasseri* from 0.1 initial OD culture observed by SEM with a magnification of 1000x, 2000x and 10000x, respectively. Yellow arrows – *C. glabrata* cells; Red arrows – *L. gasseri* cells

The effect of *L. gasseri* supernatant and of *L. gasseri* live cells in hyphae formation by *C. albicans* was also tested (Figure 37 and Appendix 22 to Appendix 38) since this is another highly relevant virulence trait attributed to this yeast species[21,44,71]. As such, *C. albicans* cells were cultivated in RPMI supplemented with FBS (fetal bovine serum) to induce filamentation and with appropriate amount of the bacterial supernatant or of *L. gasseri* live cells. Two pHs were used for the RPMI medium, pH 7 which is known to favour filamentation and pH 4 which is closer to the vaginal pH. In Figure 37 panel A are represented the *C. albicans* cells cultivated in the absence of FBS, not being observed the formation of hyphae in pH4. FBS supplementation induced, as expected, the formation of hyphae, this being more evident at pH 7 than at pH 4 (panel B Figure 37). The presence of the bacterial supernatant reduced the number of visible hyphae (Figure 37 panel C), this not being observed when only the MRS medium was added to the FBS-supplemented RPMI medium. Notably, a

similar phenotype was also obtained when *C. albicans* cells were exposed to live *L. gasseri* bacteria (panel D Figure 37).

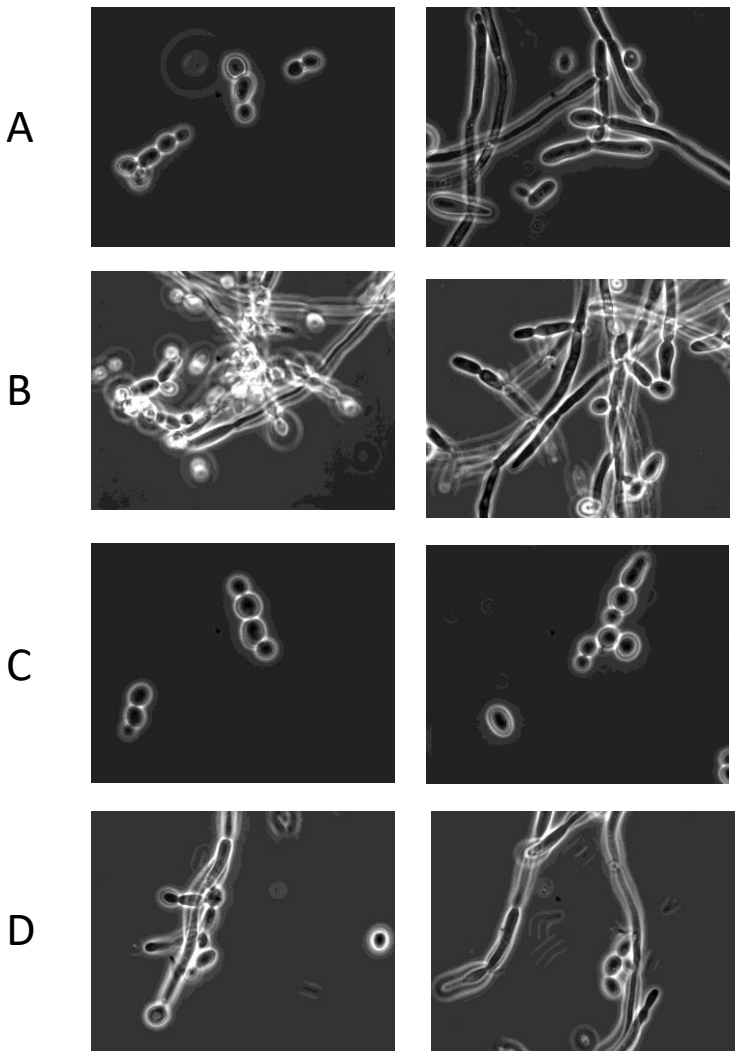

Figure 37 - Optical microscopy of *C. albicans* at 24h growth in: A) RPMI pH4 and pH7, respectively; B) RPMI pH4 and pH7 with FBS (1:10), respectively; C) RPMI pH4 and pH7 with *L. gasseri* supernatant, respectively; D) RPMI pH4 and pH7 with *L. gasseri* live cells and FBS (1:10); Observed by optical microscopy with a magnification of 1000x

Discussion

The interest in the study of the effect on the physiology of *C. albicans* or *C. glabrata* caused by the presence of Lactobacilli, has been boosted by the emerging descriptions of the essential role played by commensal bacteria in restraining the growth of vaginal pathogens. The goal of this work was to analyse the effect of *L. gasseri* and *L. jensenii* live cells on the physiology of *C. albicans* and *C. glabrata*, thus differing from much of the work that has already been performed but in species other than those indigenous to the vaginal[73,74]. The results obtained showed that both the growth rates and cellular viability of *C. albicans* SC5314 and *C. glabrata* KUE100 were reduced by the presence of *L. gasseri* and *L. jensenii*, being the decrease of the *C. glabrata* strain much more evident compared with the one obtained for *C. albicans*. The inhibitory effect was somehow expected as several works performed with *Candida* spp. and Lactobacilli had demonstrated that Lactobacilli can inhibit *Candida* spp. through several mechanisms like competition for nutrients and for adhesion sites and also the production of bacteriocin-like compounds (BLC), biosurfactants and hydrogen peroxide (H2O2)[21]. Another way by which Lactobacilli are considered to inhibit growth of *Candida* spp. is through the production and excretion of weak acids to the medium. The production of lactic acid in the co-culture setting used was observed, being demonstrated that under the microaerophilic conditions used both *L. gasseri* and *L. jensenii* produced lactic acid (in the range of 4 to 6 g/L). These levels of lactic acid produced are consistent with those obtained for *L. plantarum* (~6 g/L) after 78h of cultivation at 37ºC in MRS in anaerobic conditions[75]. It was also registered the production of ethanol by *C. glabrata* and *C. albicans* reinforcing that the experimental setting used favoured microaerophlic environment, something that is known to occur in the vaginal tract[76]. One peculiar aspect observed was the low rate of consumption of the glucose available in the MRS medium by the *L. gasseri* and *L. jensenii* cells, this being much more evident for *L. jensenii*. This difficulty in glucose consumption was exacerbated in the co-culture setting which is likely to result from a competitive effect triggered by the *Candida* cells which are using the glucose available. It is possible that the bacteria may use other compounds present in the MRS medium as carbon sources, one possibility being the many amino acids that are present in the medium. Further studies are required to better elucidate this aspect because it could pinpoint the identification of relevant mechanisms by which *Candida* and Lactobacilli compete for nutrients in the vaginal tract.

Susceptibility to the presence of the bacterial cells of a small cohort of *C. albicans* vaginal isolates was identical to the one exhibited by the laboratory strain SC5314. Differently, *C. glabrata* vaginal isolates were found to be much more tolerant to the presence of Lactobacilli than the laboratory strain KUE100. This result is interesting as it seems to suggest that during adaptation *C. glabrata* cells evolve adaptive responses to cope with the presence of the bacteria in its environment, these adaptive responses being stable and most probably resulting from alterations in the genomic sequence. To better understand this matter it will be necessary to obtain more information with a broader cohort of vaginal isolates and eventually also use strains from other origins as well (e.g. from the GI tract). Genome sequencing of the vaginal isolates may also provide additional information on

this matter, although the genes mediating *C. glabrata* survival in the presence of Lactobacilli remain to be identified.

Growth, ability to form biofilms and to induce filamentation by *C. albicans* and *C. glabrata* was found to be reduced by the presence of *L. gasseri* and also of a supernatant obtained from a culture where this bacterium was cultivated for 48h. Similarly, supernatants of *L. crispatus* cultures were also found to reduce filamentation in *C. albicans*[71]. The fact that inhibitory effect is observed either using live cells and the culture supernatant is consistent with the accumulation in the medium of one or more inhibitory compounds that are secreted by the bacteria. Surfactants or bacteriocines are some of the inhibitory compounds that could underlie this. No genes related with both synthesis of surfactants or bacteriocins were found to be differently expressed in the *L. gasseri-C. glabrata* co-culture, based on the data from the RNA-seq analysis performed; however, it is important to say that most genes involved in the production of these two types of compounds have not been properly identified in this bacterium. Further studies are required to investigate this. It was also interesting to note that both *C. albicans* and *C. glabrata* were able to form mixed biofilms with *L. gasseri* and *L. jensenii*. Other bacteria have also been able to form biofilms with *C. albicans* including *Streptococcus gordonii* and *Staphylococcus epidermidis*[77,78]. Further studies are required to clarify the extent at which this ability to form mixed biofilms could contribute for the interaction between Lactobacilli and *Candida* species although the fact that our results show a close contact between the two in these biofilm structures suggest that it could be a matter of relevance.

To obtain further insights into the responses evolved by *C. glabrata* to the presence of Lactobacilli an RNA-seq analysis was performed using the co-culture setting that was implemented in this work. Unfortunately the analysis of these results was highly time-consuming and the results were only rendered available on mid-November, which prevented a thorough analysis to be shown in this thesis. Nevertheless, it was possible to observe a significant alteration in the genomic expression of *C. glabrata* in the presence of the bacteria, with almost 700 differently expressed genes (2h and 8h) when in co-culture. It was also interesting to see that the differences were considerably higher at the higher time-point of 8h, probably due to the increasing number of bacteria present in the co-culture which potentiates the inhibitory effect. It was also interesting to observe that the top 10 up-regulated genes at 8h (Table 13) encode almost all proteins of uncharacterized function in *C. glabrata*, thereby remaining to be established their role in assuring competitiveness of *C. glabrata* towards *L. gasseri* cells. In the case of *L. gasseri* almost all the top 10 up-regulated genes (at 8h) have known function, mostly related with transporters and membrane proteins (Table 13).

Table 13 – Top 10 up-regulated genes of *C. glabrata* and *L. gasseri* at 8h of co-culture compared with the respective single-culture

Up-regulated *C. glabrata* genes	Up-regulated *L. gasseri* genes and gene function
CAGL0B00792g	gene1403 (ABC type transporter)
CAGL0A02255g	gene28 (Uncharacterized)
CAGL0A01804g	gene217 (ABC transporter)
CAGL0E05522g	gene183 (Uncharacterized)
CAGL0F00407g	gene216 (Sugar ABC transporter)
CAGL0I06006g	gene215 (Maltose ABC transporter)
CAGL0B00352g	gene1404 (ABC transporter ATP-binding protein)
CAGL0F02563g	gene39 (DNA-damage-inducible protein J)
CAGL0M12430g	gene214 (ABC transporter)
CAGL0E02673g	gene182 (Uncharacterized)

Another interesting result that comes from this work concerns the observation that the expression of *CgHaa1* plays a critical role in determining susceptibility of *C. glabrata* cells to the presence of *L. gasseri*. Although *CgHaa1* was found to provide protection against acetic acid and also lactic acid[54,57], it is unlikely that the herein registered protective effect relates with those previous described functions because we could not detect significant amounts of acetic acid in the co-culture and the amount of lactic acid was far below the level found to be inhibitory for *C. glabrata* and *C. albicans*, at least in vitro[57]. Thus, it is possible that the protective effect exerted by *CgHaa1* results from a new biological role attributable to this protein. The DNA motif serving as a binding site for *CgHaa1* is not known, however, for its close orthologue ScHaa1 this was established as being 5'-(G/C)(A/C)GG(G/C)-3'[79]. *CgHaa1* and *ScHaa1* share a high degree of homology at the level of the DNA binding domain and therefore it is conceivable to hypothesize that they could recognize similar binding sites[57]. In this sense, a search for the *ScHaa1* binding site over the set of genes found to be up-regulated in response to the presence of *L. gasseri* was undertaken as an indirect mean of assessing the relevance of *CgHaa1* in the remodelling of *C. glabrata* genomic expression in a co-culture context. The results obtained showed that this motif is present in 49 of the 53 genes up-regulated after 2h of co-culture and in 413 out of 429 genes found to be up-regulated after 8h of co-culture. Necessarily, these high numbers may also reflect the degeneracy associated to the *ScHaa1* binding site, but they might also point to a critical role of *CgHaa1* in the control of *C. glabrata* transcriptome-wide response to *L. gasseri*. It is important to highlight that at least 10 *C. glabrata* genes up-regulated at 8h were already described as *CgHaa1* targets[57]. Further studies are now required to fully understand the extent of *CgHaa1* relevance in assuring competitiveness of *C. glabrata* in the vaginal microbiome.

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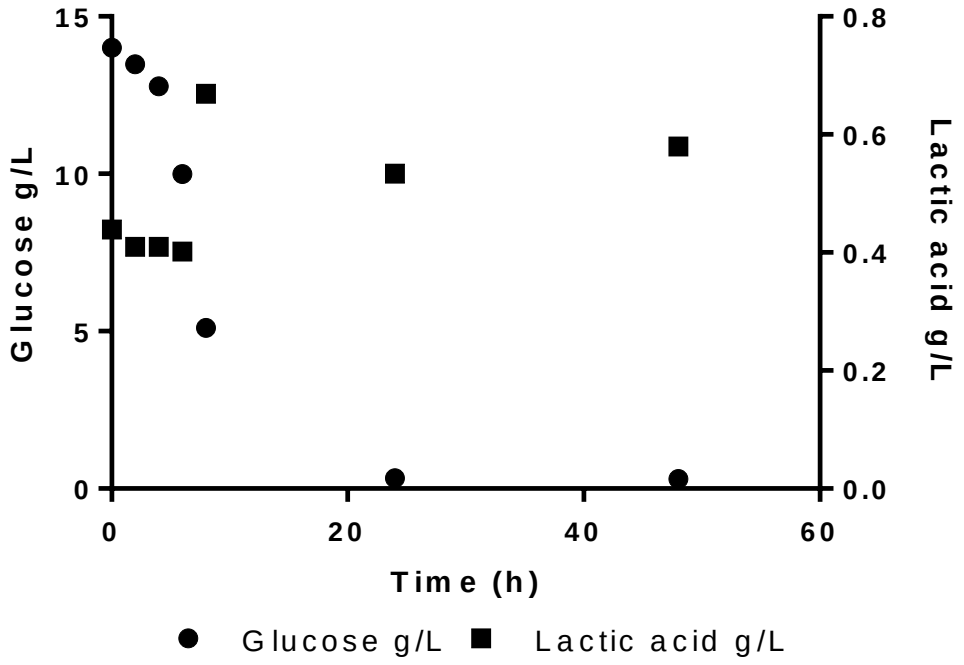
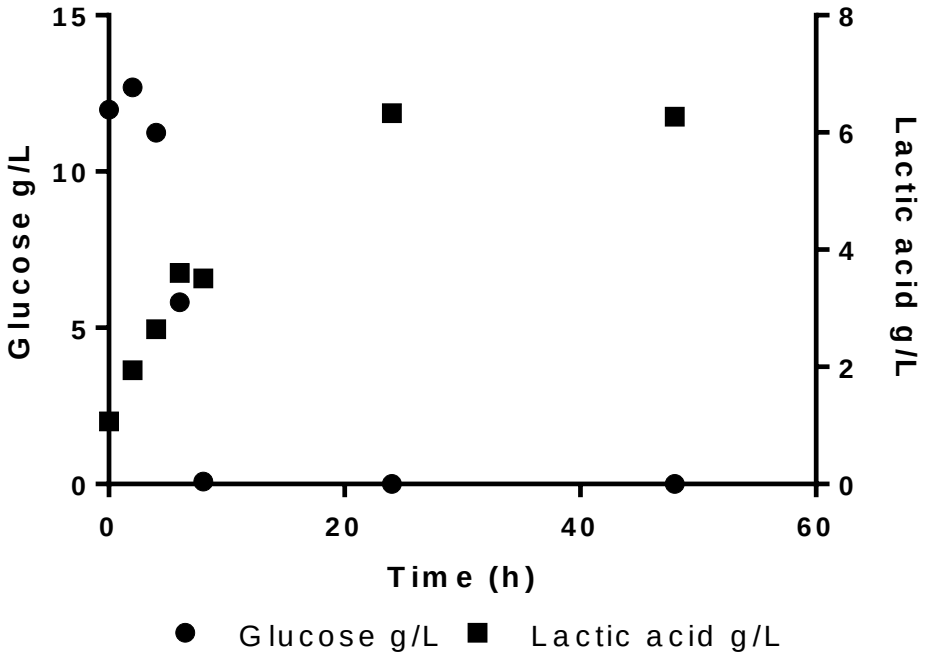
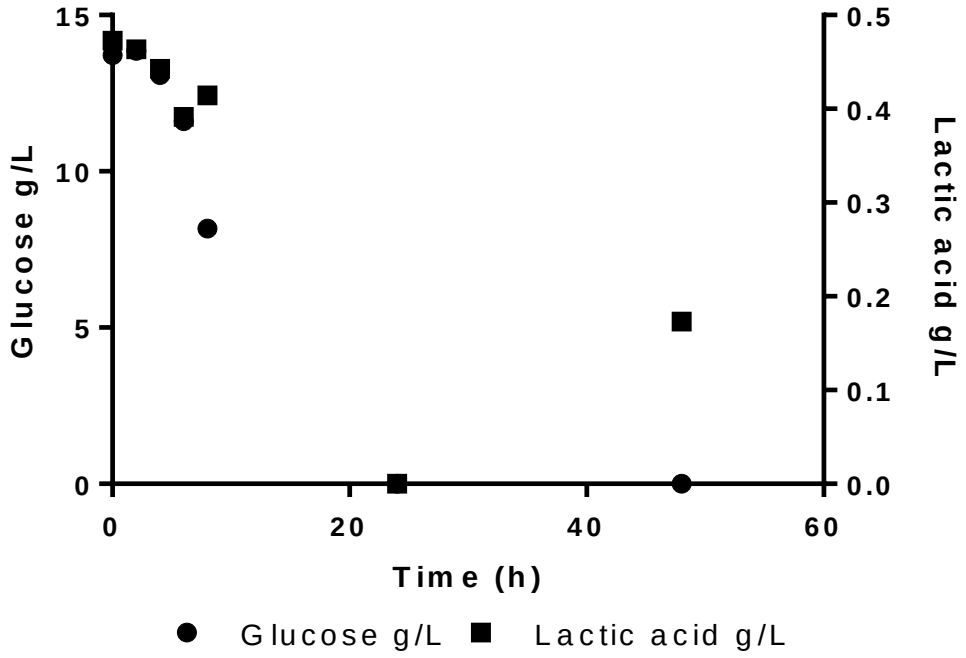
Appendixes



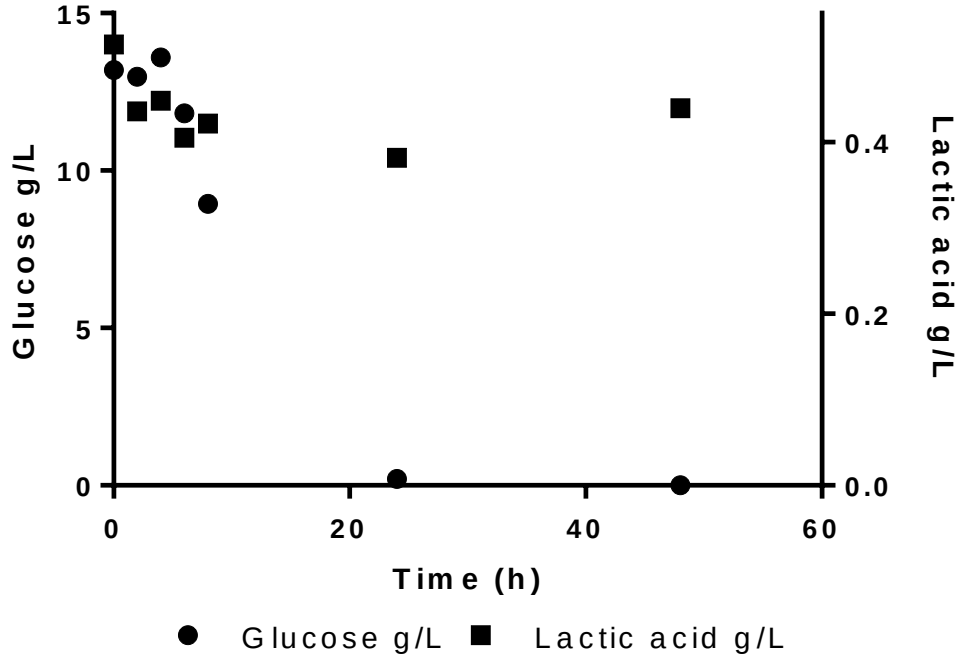
Appendix 1 - Glucose consumption and lactic acid production by *C. glabrata* BG2 single-culture.



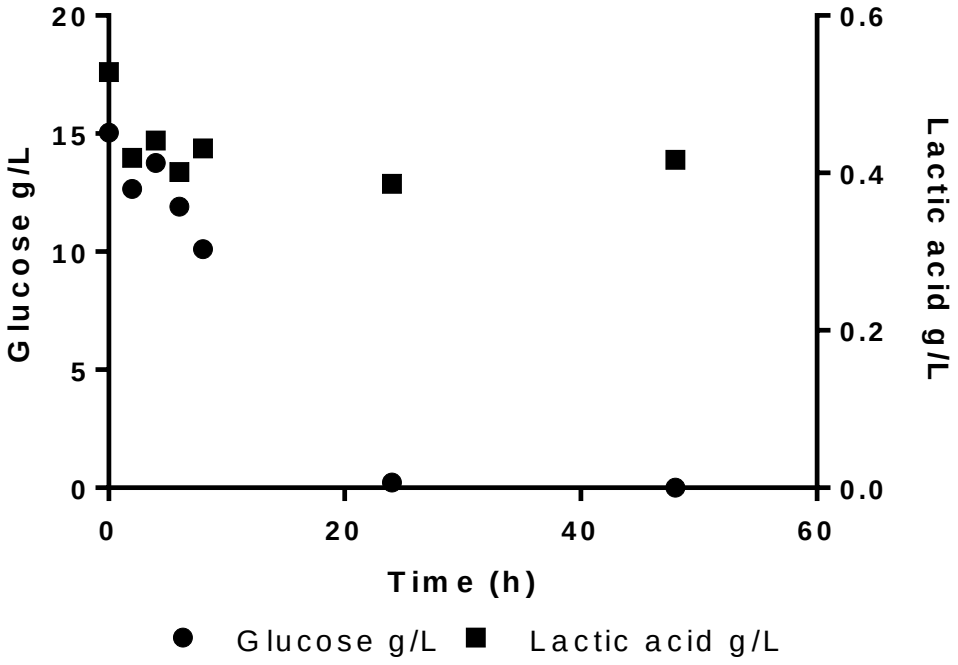
Appendix 2 - Glucose consumed and lactic acid produced in co-culture with *C. glabrata* BG2 and *L. gasseri* (1:4)



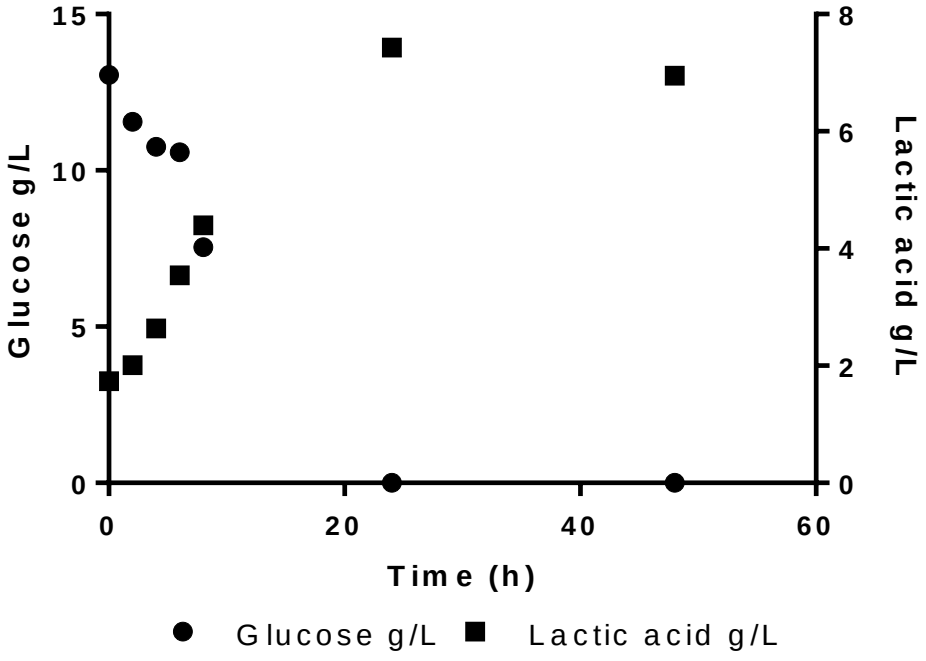
Appendix 3 - Glucose consumption and lactic acid production by *C. albicans* VG216 single-culture.



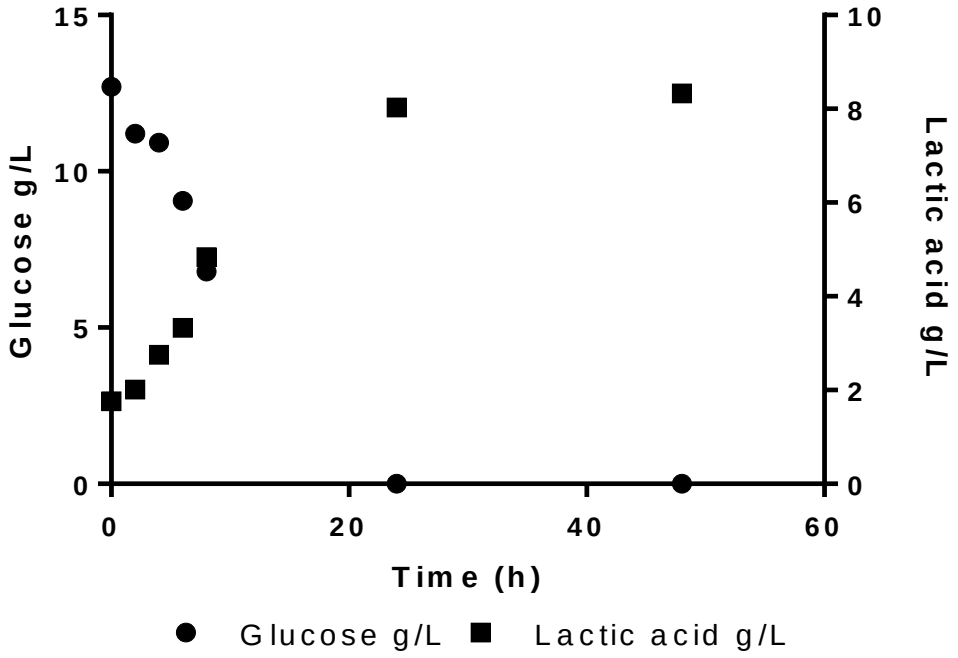
Appendix 4 - Glucose consumption and lactic acid production by *C. albicans* VG217 single-culture.



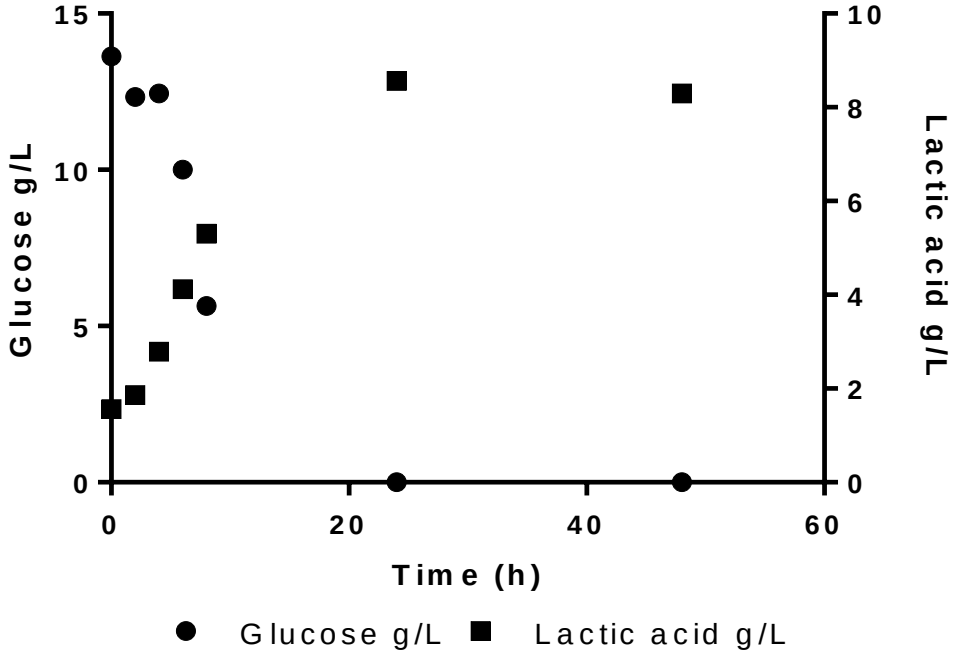
Appendix 5 - Glucose consumption and lactic acid production by *C. albicans* VG485 single-culture.



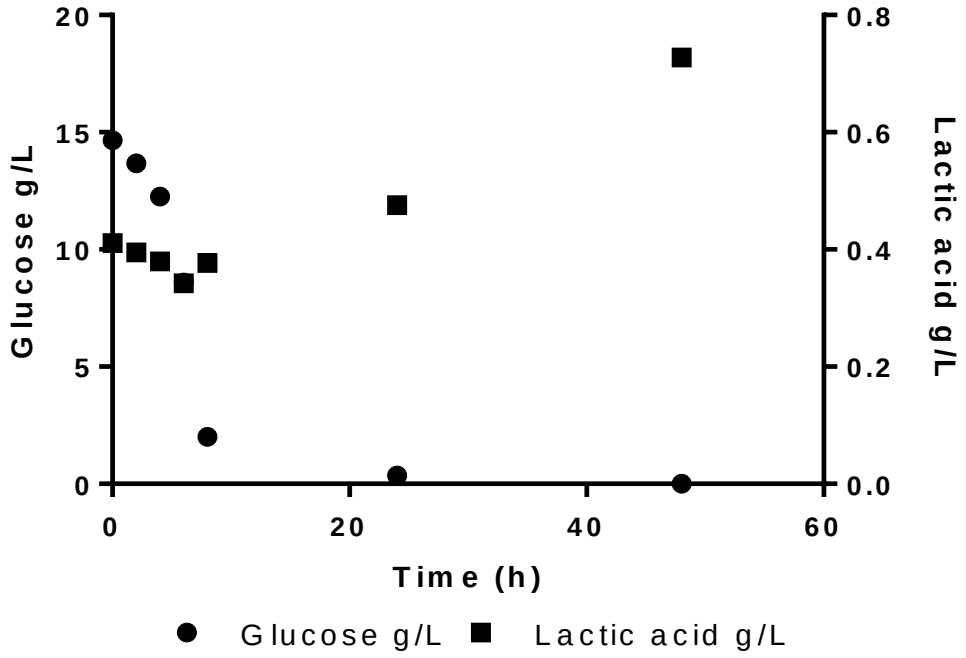
Appendix 6 - Glucose consumed and lactic acid produced in co-culture with *C. albicans* VG216 and *L. gasseri* (1:4)



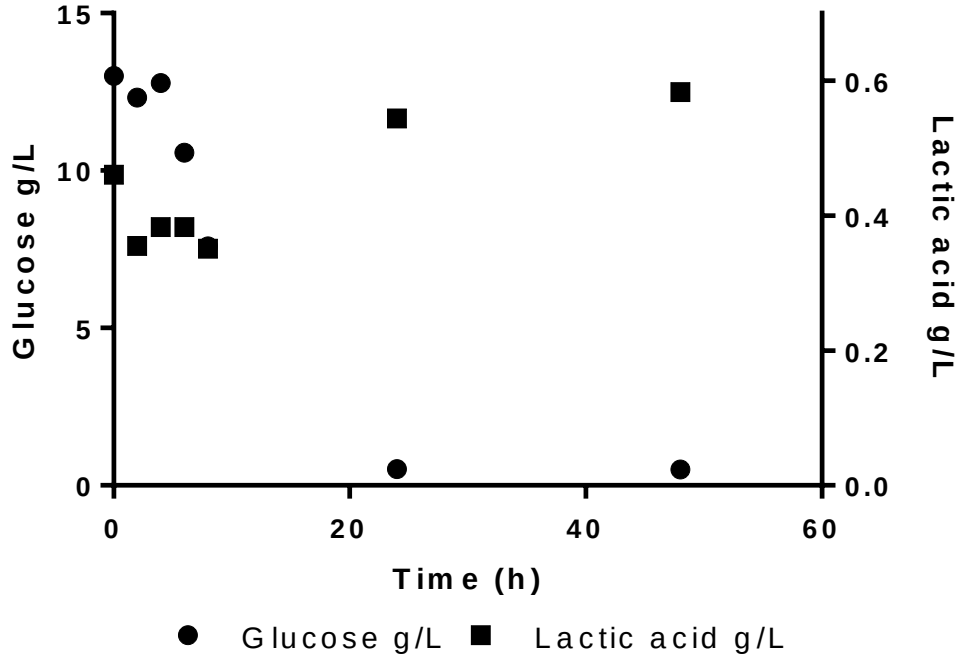
Appendix 7 - Glucose consumed and lactic acid produced in co-culture with *C. albicans* VG217 and *L. gasseri* (1:4)



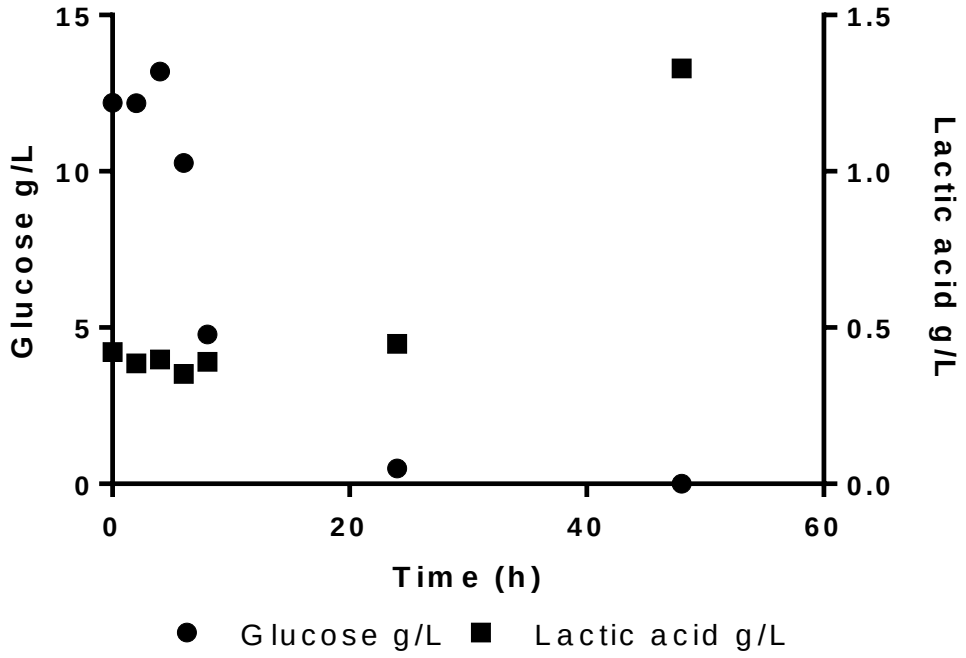
Appendix 8 - Glucose consumed and lactic acid produced in co-culture with *C. albicans* VG485 and *L. gasseri* (1:4)



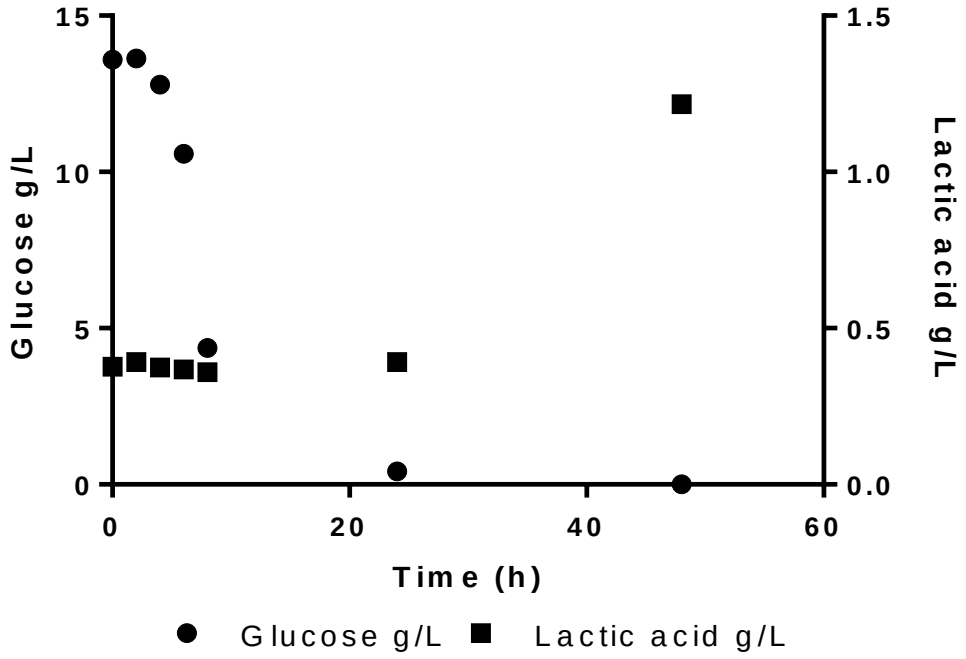
Appendix 9 - Glucose consumption and lactic acid production by *C. glabrata* VG99 single-culture.



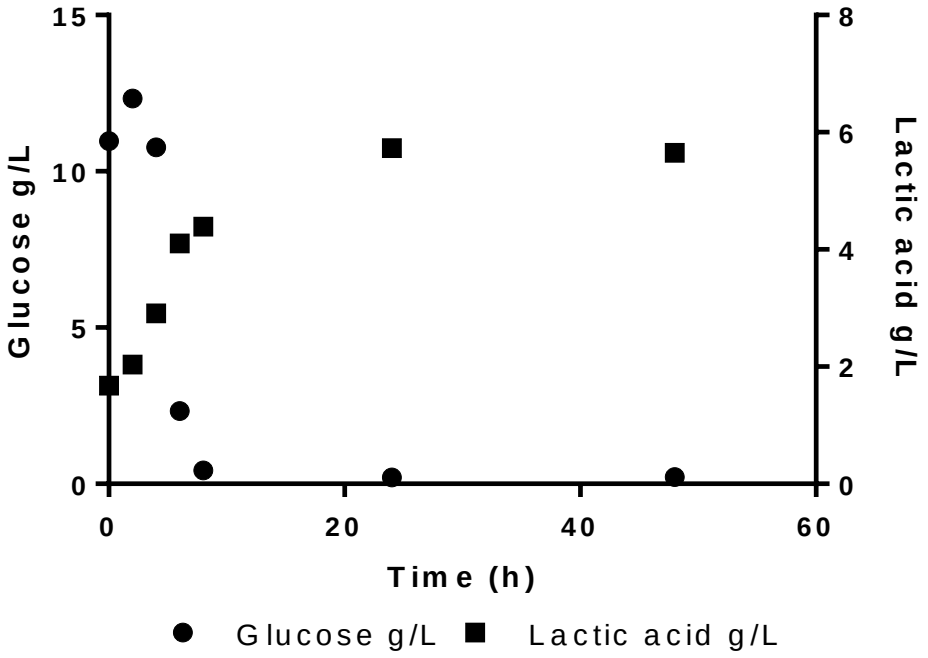
Appendix 10 - Glucose consumption and lactic acid production by *C. glabrata* VG281 single-culture.



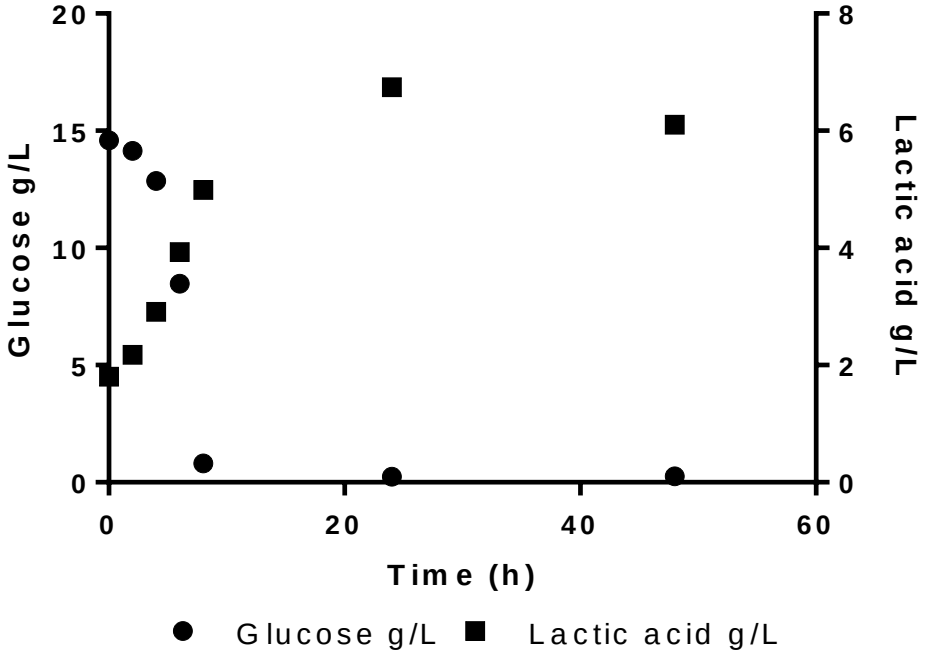
Appendix 11 - Glucose consumption and lactic acid production by *C. glabrata* VG49 single-culture.



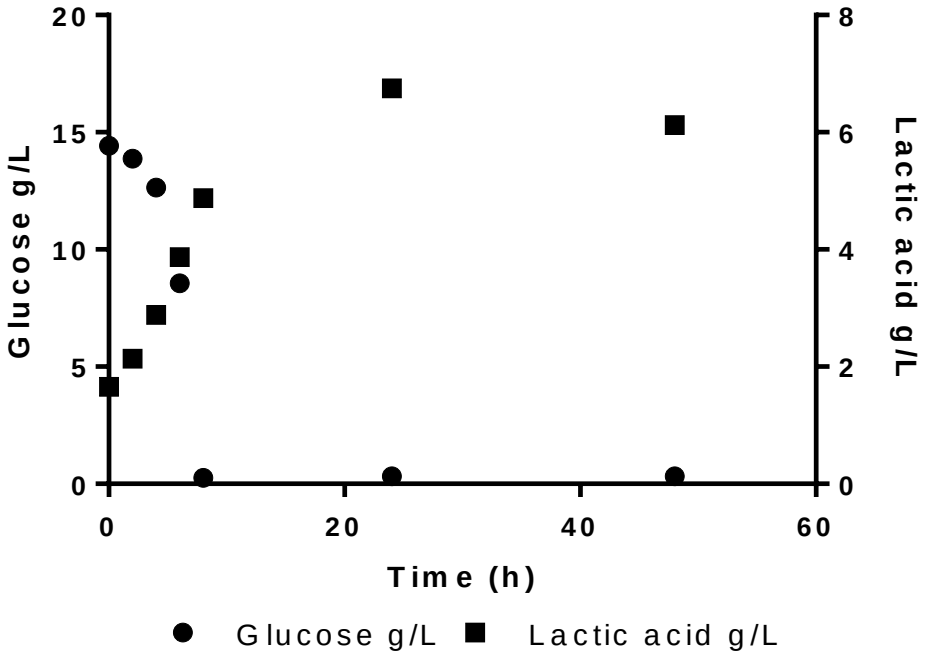
Appendix 12 - Glucose consumption and lactic acid production by *C. glabrata* VG216 single-culture.



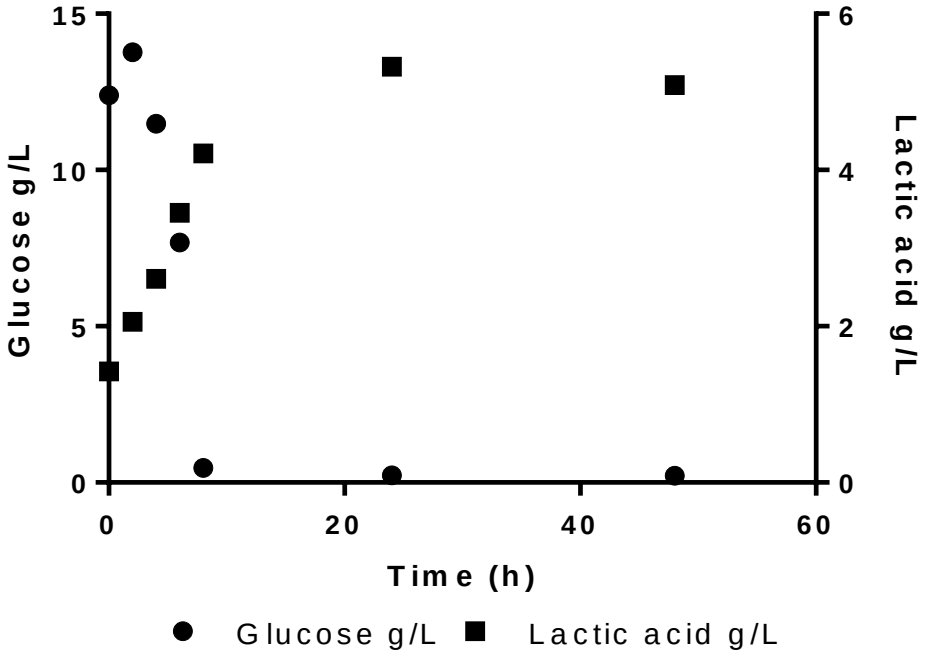
Appendix 13 - Glucose consumed and lactic acid produced in co-culture with *C. glabrata* VG99 and *L. gasseri* (1:4)



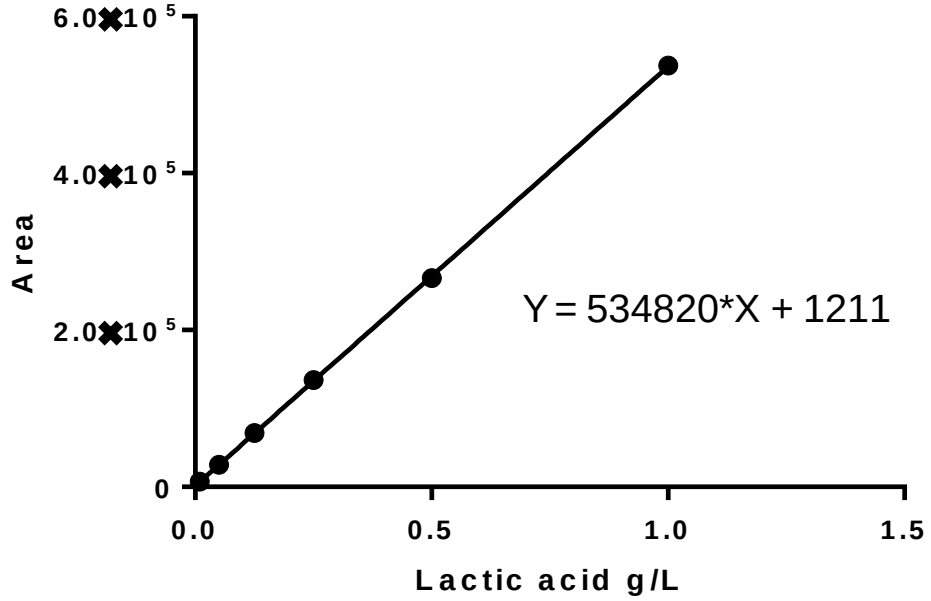
Appendix 14 - Glucose consumed and lactic acid produced in co-culture with *C. glabrata* VG281 and *L. gasseri* (1:4)



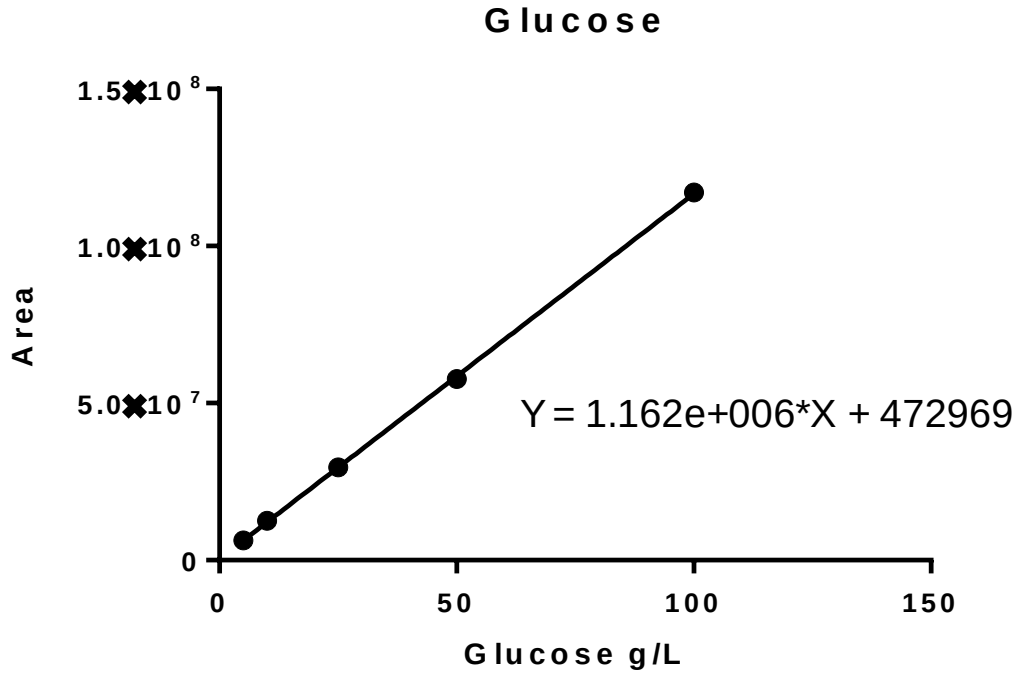
Appendix 15 - Glucose consumed and lactic acid produced in co-culture with *C. glabrata* VG49 and *L. gasseri* (1:4)



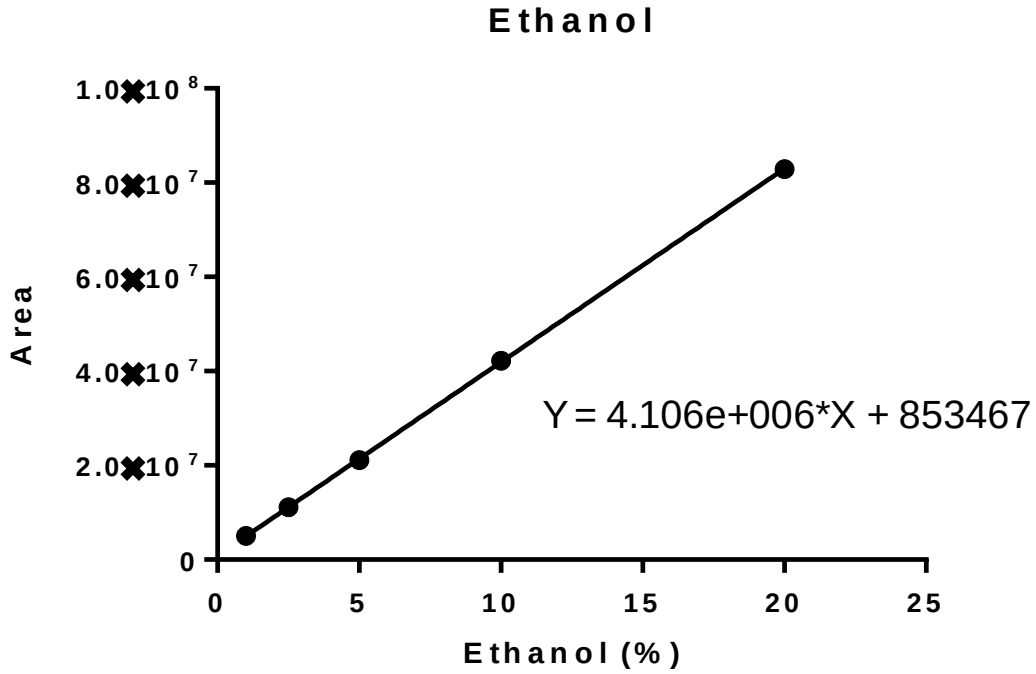
Appendix 16 - Glucose consumed and lactic acid produced in co-culture with *C. glabrata* VG216 and *L. gasseri* (1:4)



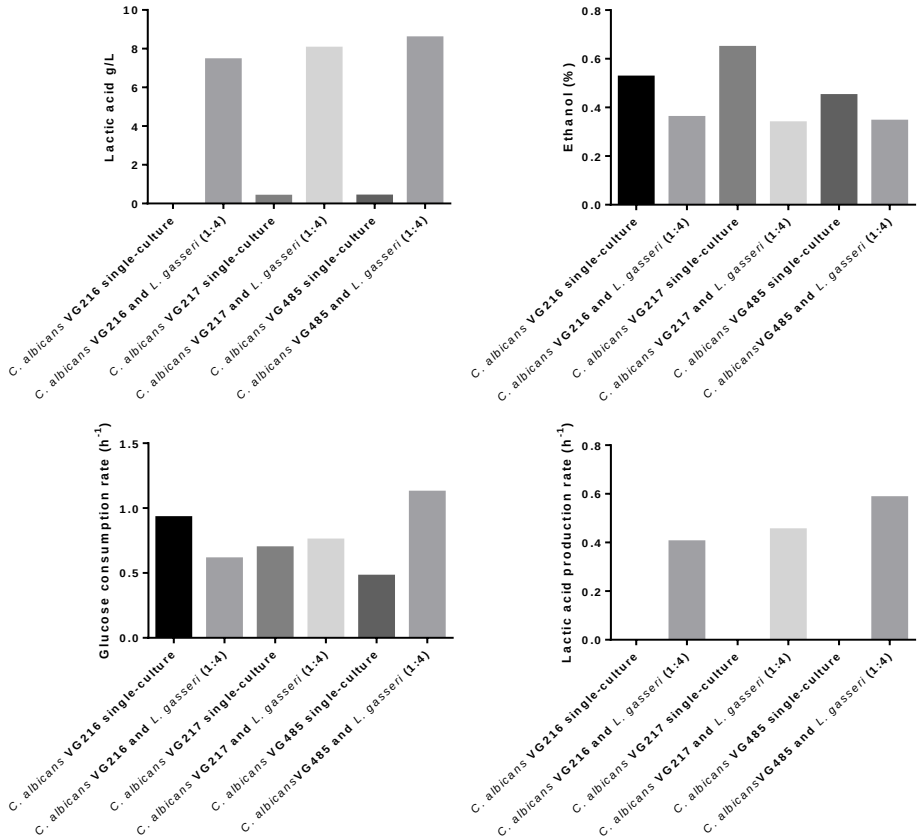
Appendix 17 – Lactic acid calibration curve



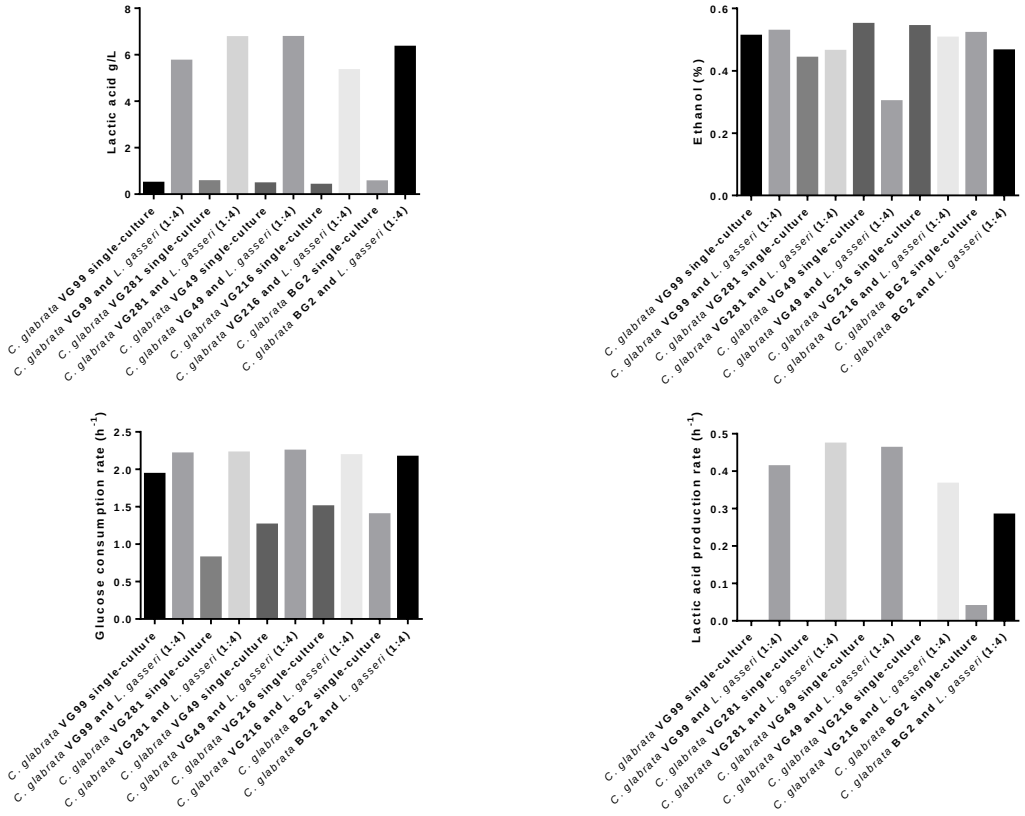
Appendix 18 – Glucose calibration curve



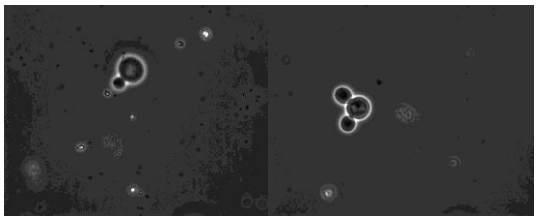
Appendix 19 – Ethanol calibration curve



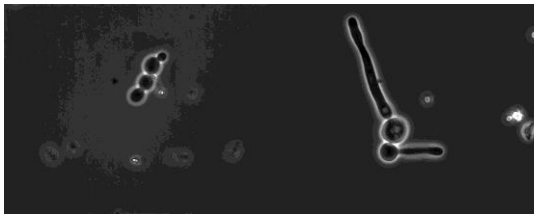
Appendix 20 - Lactic acid concentration at 24h, final ethanol concentration, glucose consumption rate and lactic acid production rate for single-culture and co-culture of *C. albicans* vaginal isolates.



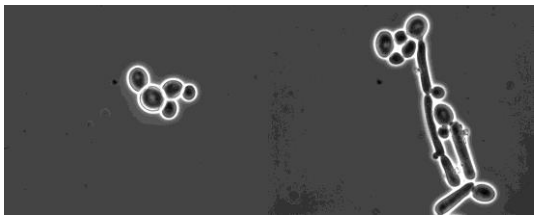
Appendix 21 - Lactic acid concentration at 24h, final ethanol concentration, glucose consumption rate and lactic acid production rate for single-culture and co-culture of *C. glabrata* vaginal isolates and BG2.



Appendix 22 - Optical microscopy of *C. albicans* at 30 minutes growth in MRS medium, MRS medium and FBS (fetal bovine serum), respectively.

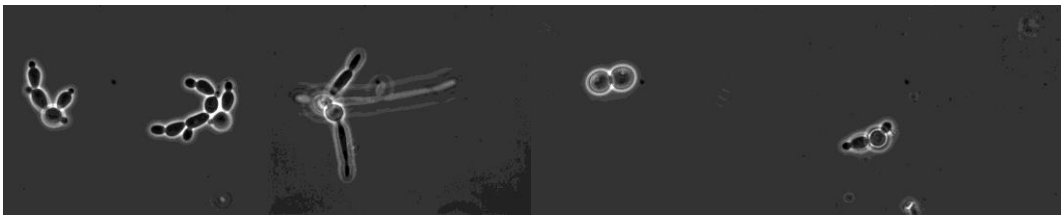


Appendix 23 - Optical microscopy of *C. albicans* at 2h growth in MRS medium, MRS medium and FBS (fetal bovine serum), respectively.

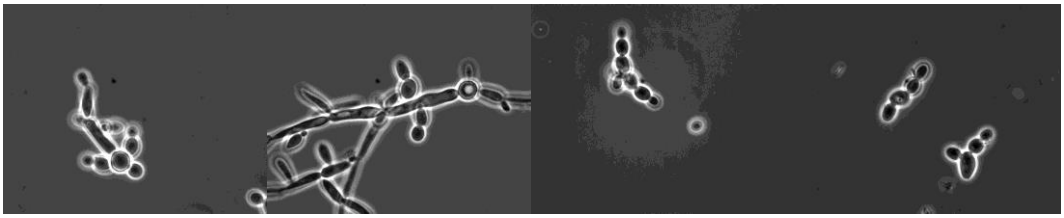


Appendix 24 - Optical microscopy of *C. albicans* at 2h growth in MRS medium, MRS medium and FBS (fetal bovine serum), respectively.

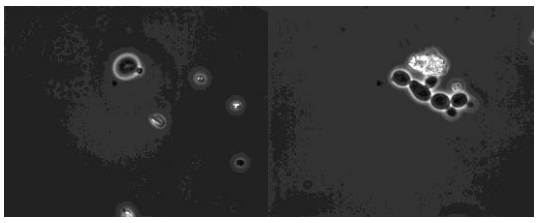
Appendix 25 - Optical microscopy of *C. albicans* at 30 minutes growth in RPMI pH4 medium, RPMI pH4 medium and FBS (fetal bovine serum), RPMI pH4 medium and *L. gasseri* supernatant and RPMI pH4 medium, *L. gasseri* supernatant and FBS, respectively.



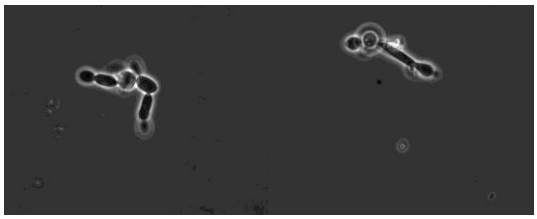
Appendix 26 - Optical microscopy of *C. albicans* at 2h growth in RPMI pH4 medium, RPMI pH4 medium and FBS (fetal bovine serum), RPMI pH4 medium and *L. gasseri* supernatant and RPMI pH4 medium, *L. gasseri* supernatant and FBS, respectively.



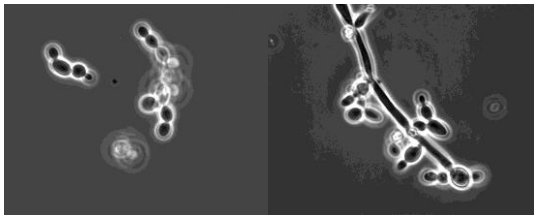
Appendix 27 - Optical microscopy of *C. albicans* at 6h growth in RPMI pH4 medium, RPMI pH4 medium and FBS (fetal bovine serum), RPMI pH4 medium and *L. gasseri* supernatant and RPMI pH4 medium, *L. gasseri* supernatant and FBS, respectively.



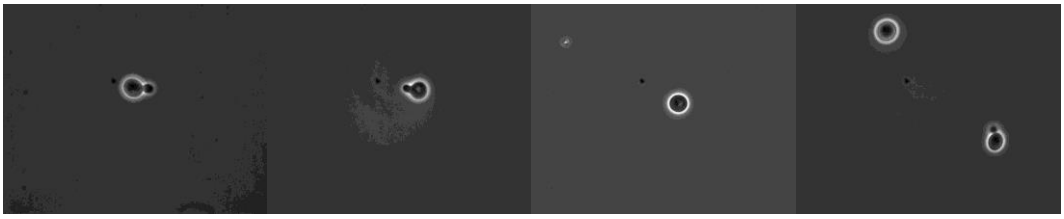
Appendix 28 - Optical microscopy of *C. albicans* at 30 minutes growth in RPMI pH4 and MRS medium and RPM pH 4 medium, MRS medium and FBS, respectively.



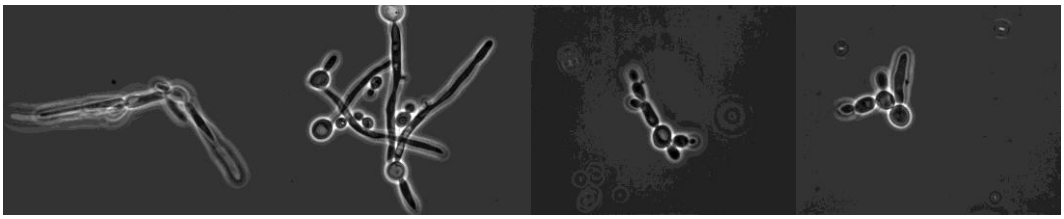
Appendix 29 - Optical microscopy of *C. albicans* at 2h growth in RPMI pH4 and MRS medium and RPM pH 4 medium, MRS medium and FBS, respectively.



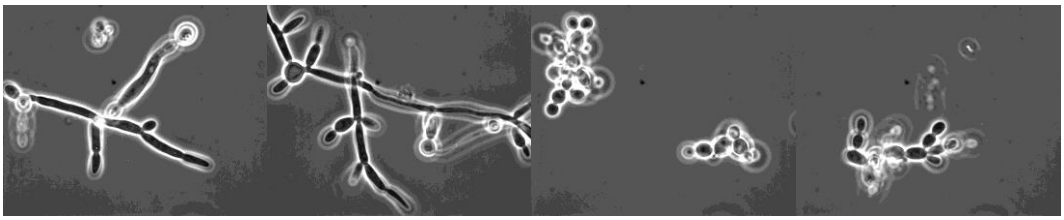
Appendix 30 - Optical microscopy of *C. albicans* at 6h growth in RPMI pH4 and MRS medium and RPM pH 4 medium, MRS medium and FBS, respectively.



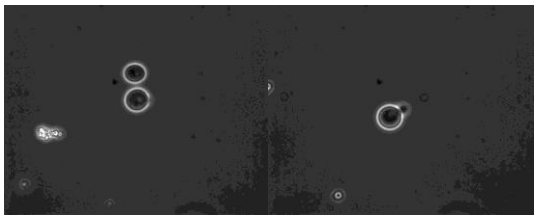
Appendix 31 - Optical microscopy of *C. albicans* at 30 minutes growth in RPMI pH7 medium, RPMI pH7 medium and FBS (fetal bovine serum) RPMI pH7 medium and *L. gasseri* supernatant and RPMI pH7 medium, *L. gasseri* supernatant and FBS, respectively.



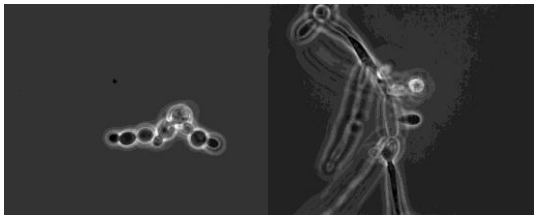
Appendix 32 - Optical microscopy of *C. albicans* at 2h growth in RPMI pH7 medium, RPMI pH7 medium and FBS (fetal bovine serum) RPMI pH7 medium and *L. gasseri* supernatant and RPMI pH7 medium, *L. gasseri* supernatant and FBS, respectively.



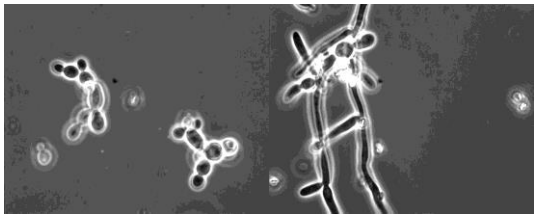
Appendix 33 - Optical microscopy of *C. albicans* at 6h growth in RPMI pH7 medium, RPMI pH7 medium and FBS (fetal bovine serum) RPMI pH7 medium and *L. gasseri* supernatant and RPMI pH7 medium, *L. gasseri* supernatant and FBS, respectively.



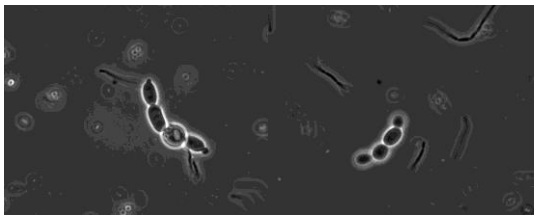
Appendix 34 - Optical microscopy of *C. albicans* at 30 minutes growth in RPMI pH7 and MRS medium and RPM pH7 medium, MRS medium and FBS, respectively.



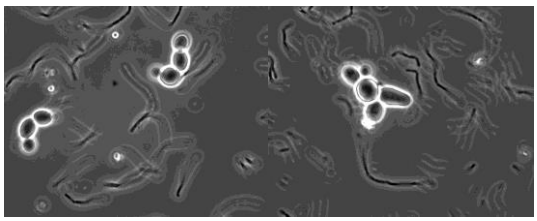
Appendix 35 - Optical microscopy of *C. albicans* at 2h growth in RPMI pH7 and MRS medium and RPM pH7 medium, MRS medium and FBS, respectively.



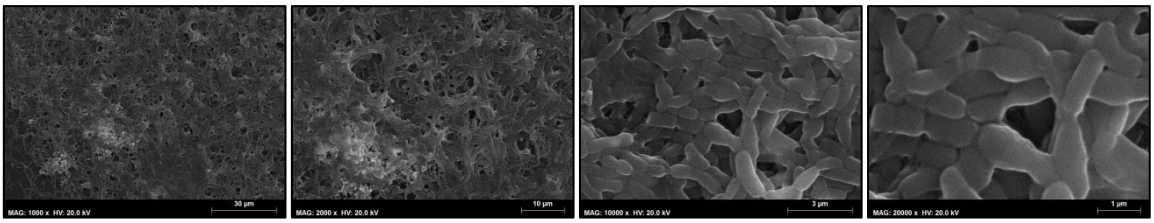
Appendix 36 - Optical microscopy of *C. albicans* at 6h growth in RPMI pH7 and MRS medium and RPM pH7 medium, MRS medium and FBS, respectively.



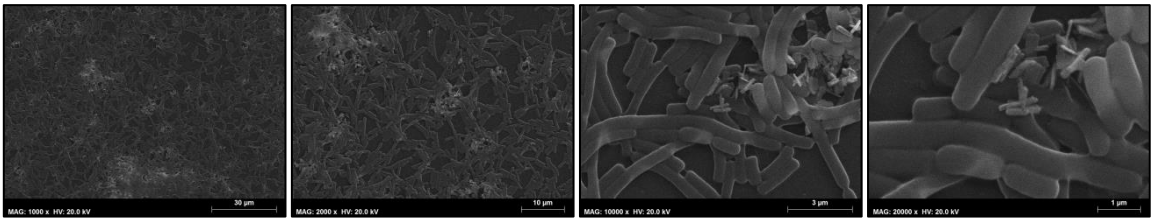
Appendix 37 - Optical microscopy of *C. albicans* at 2h growth in MRS medium and *L. gasseri* live cells, and MRS medium, *L. gasseri* live cells and FBS, respectively.



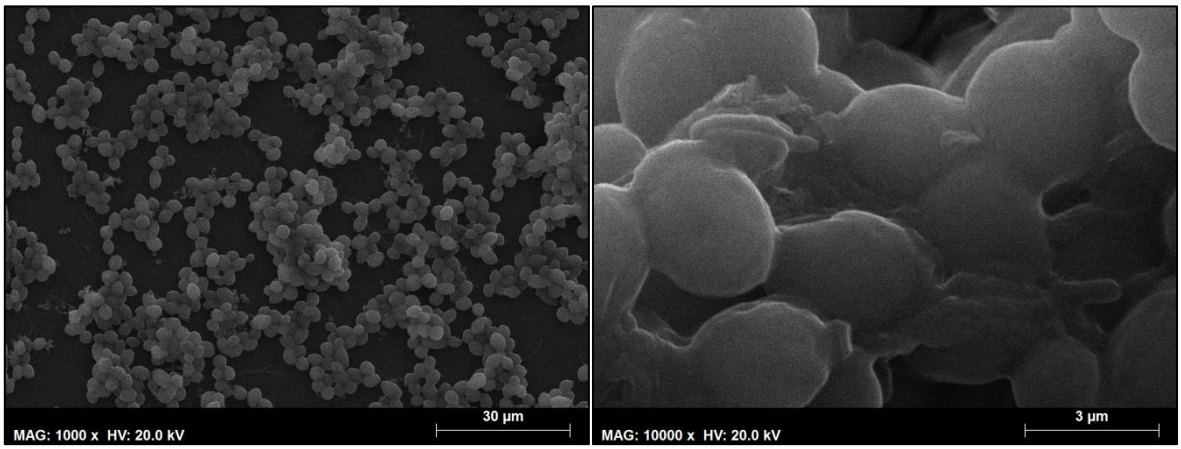
Appendix 38 - Optical microscopy of *C. albicans* at 6h growth in MRS medium and *L. gasseri* live cells, and MRS medium, *L. gasseri* live cells and FBS, respectively.



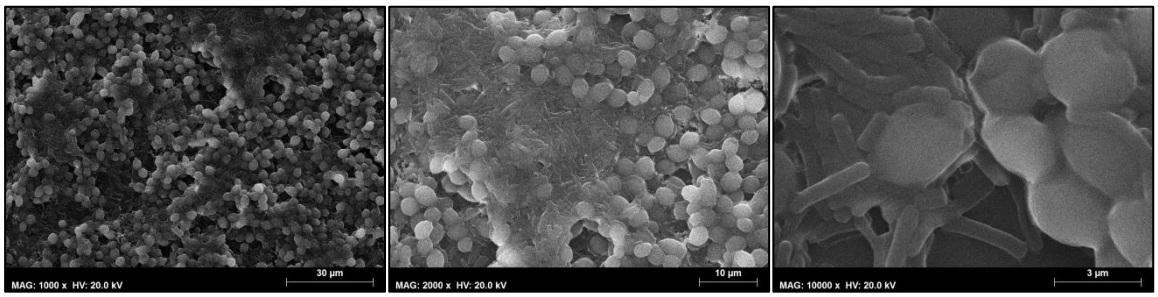
Appendix 39 – *L. jensenii* cells from 0.2 initial OD culture observed by SEM with a magnification of 1000x, 2000x, 10000x and 20000x, respectively



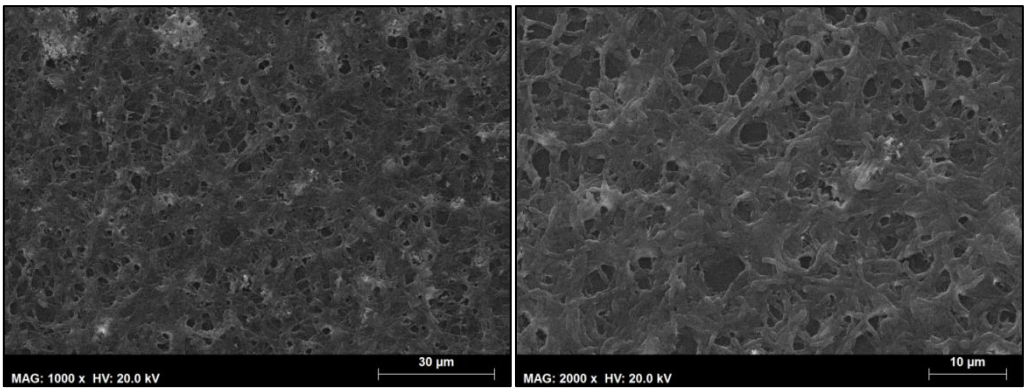
Appendix 40 - *L. gasseri* cells from 0.2 initial OD culture observed by SEM with a magnification of 1000x, 2000x, 10000x and 20000x, respectively



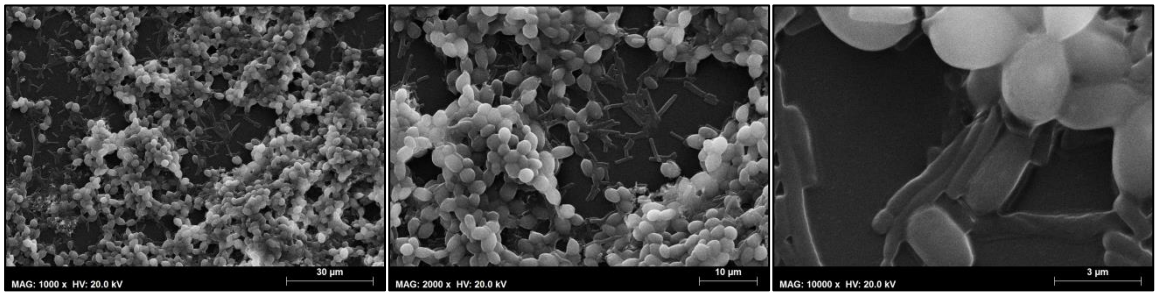
Appendix 41 - Cells from *C. albicans* and *L. jensenii* from 0.2 initial OD culture observed by SEM with a magnification of 1000x and 10000x, respectively



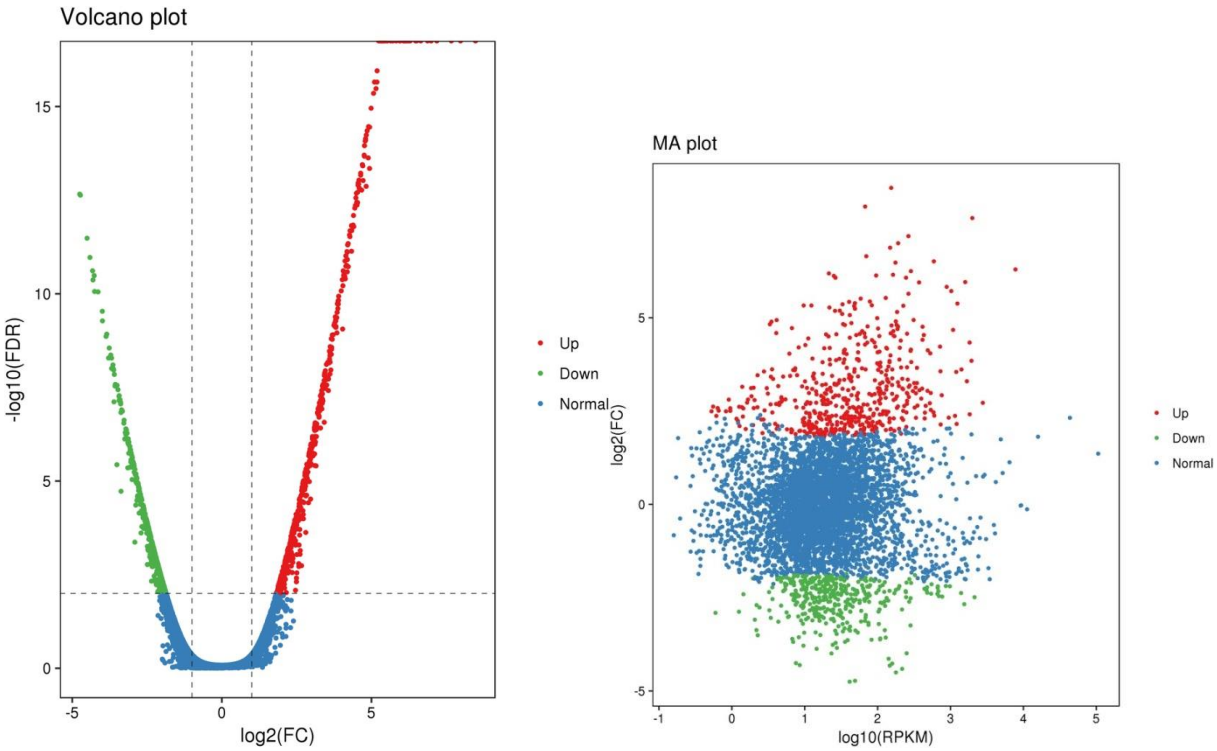
Appendix 42 – Cells from *C. albicans* and *L. gasseri* from 0.2 initial OD culture observed by SEM with a magnification of 1000x, 2000x and 10000x, respectively



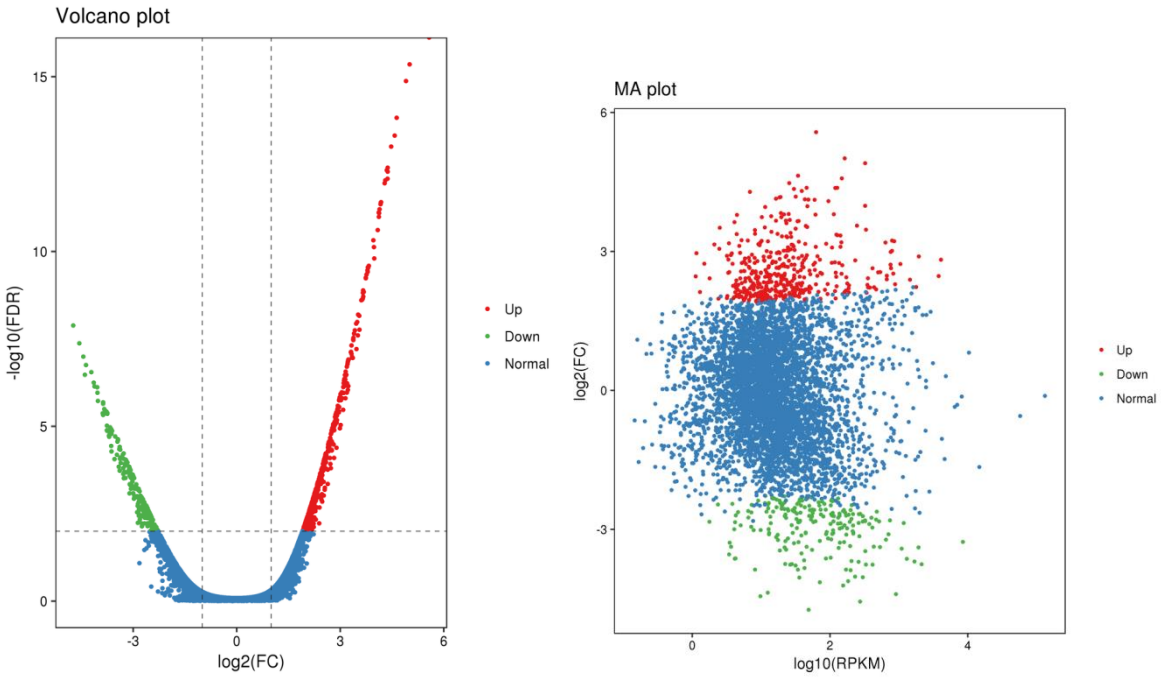
Appendix 43 – Cells from *C. glabrata* and *L. jensenii* from 0.2 initial OD culture observed by SEM with a magnification of 1000x and 2000x, respectively



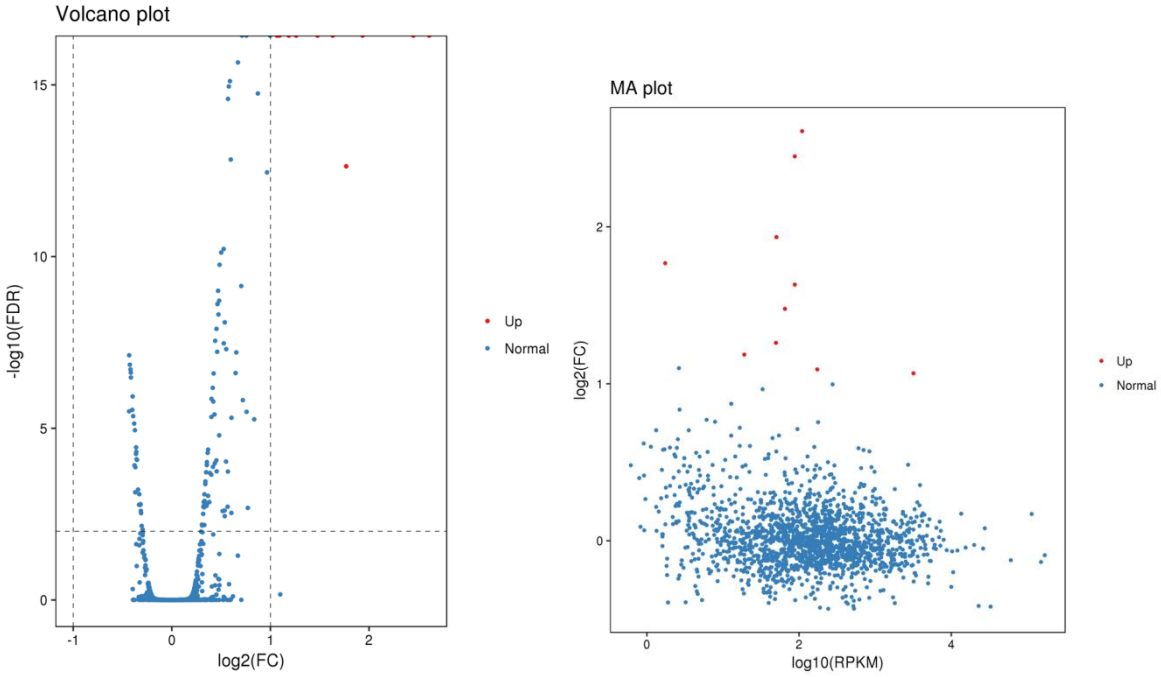
Appendix 44 – Cells from *C. glabrata* and *L. gasseri* from 0.2 initial OD culture observed by SEM with a magnification of 1000x, 2000x and 10000x, respectively



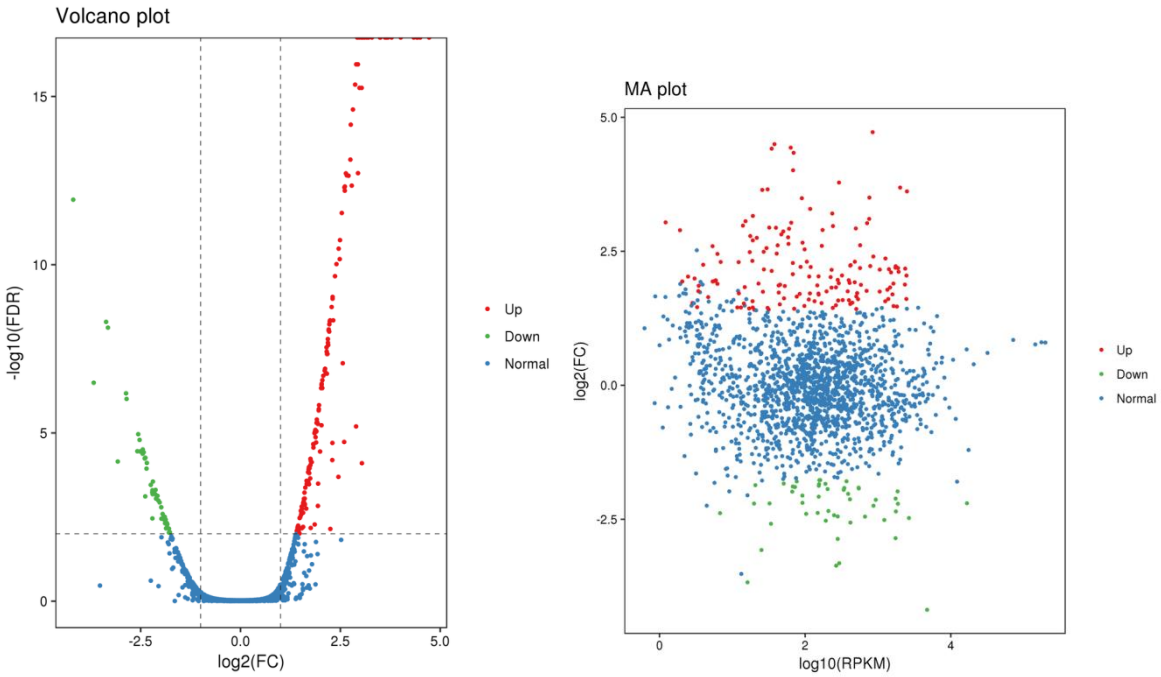
Appendix 45 - Volcano plot (left) and MA plot (right) of differentially expressed genes for Cg (single 2h) vs. Cg (co-culture 2h)



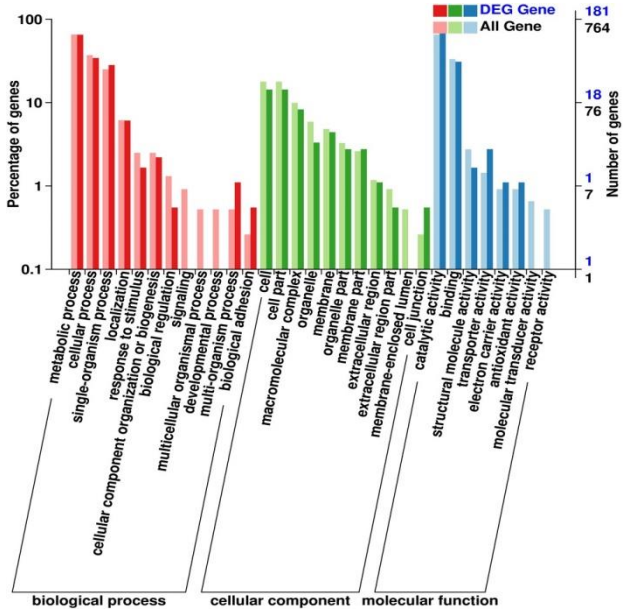
Appendix 46 - Volcano plot (left) and MA plot (right) of differentially expressed genes for Cg (single 2h) vs. Cg (single 8h)



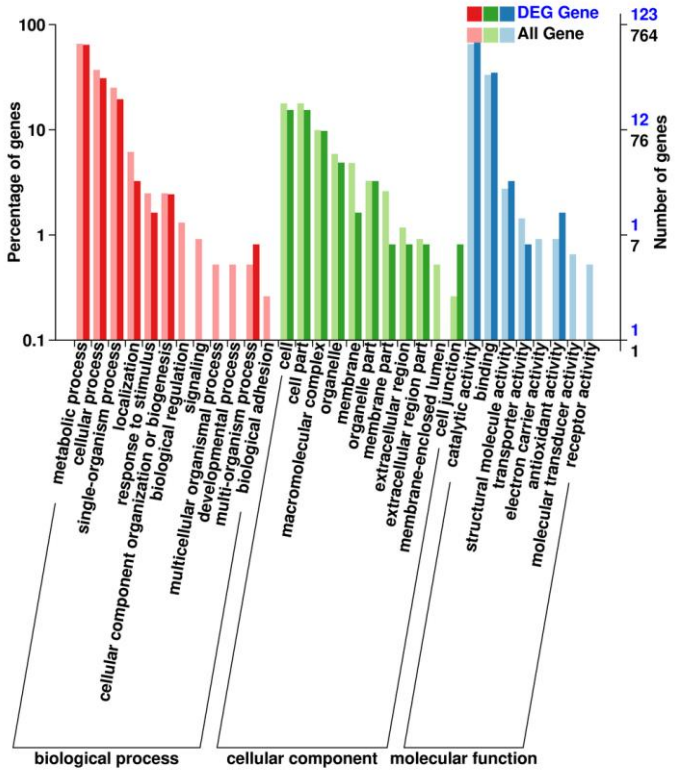
Appendix 47 - Volcano plot (left) and MA plot (right) of differentially expressed genes for Lg (single 2h) vs. Lg (single 8h)



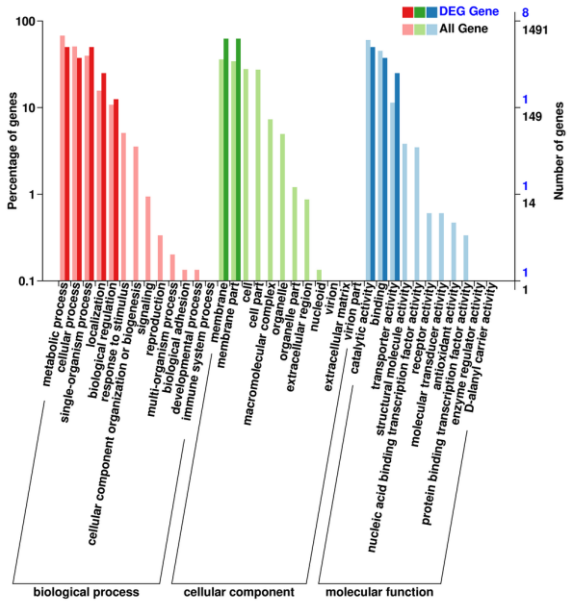
Appendix 48 - Volcano plot (left) and MA plot (right) of differentially expressed genes for Lg (single 8h) vs. Lg (co-culture 8h)



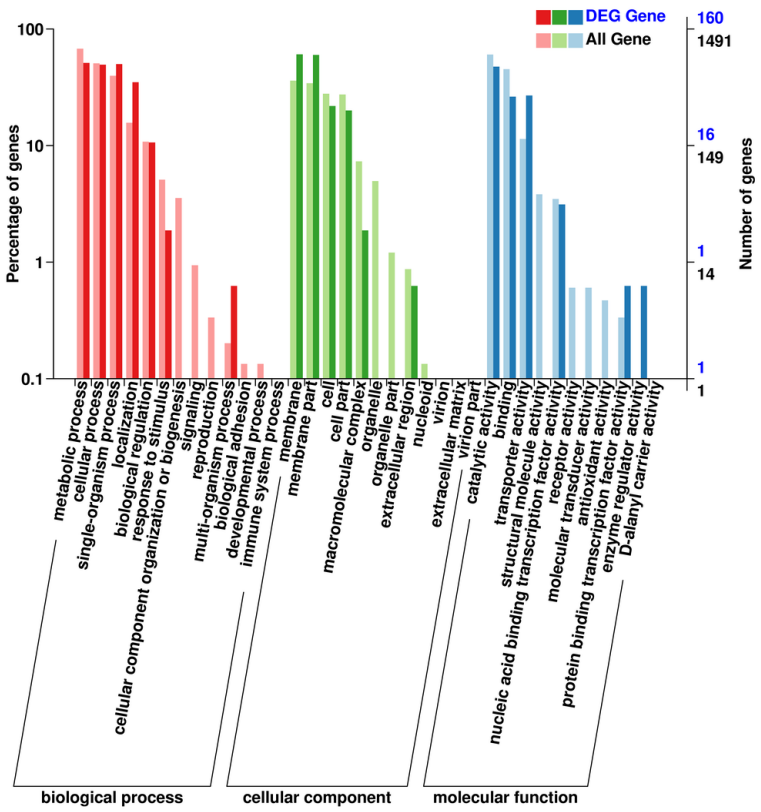
Appendix 49 - Statistics results of GO annotation for Cg (single 2h) vs. Cg (single 8h) DEGs



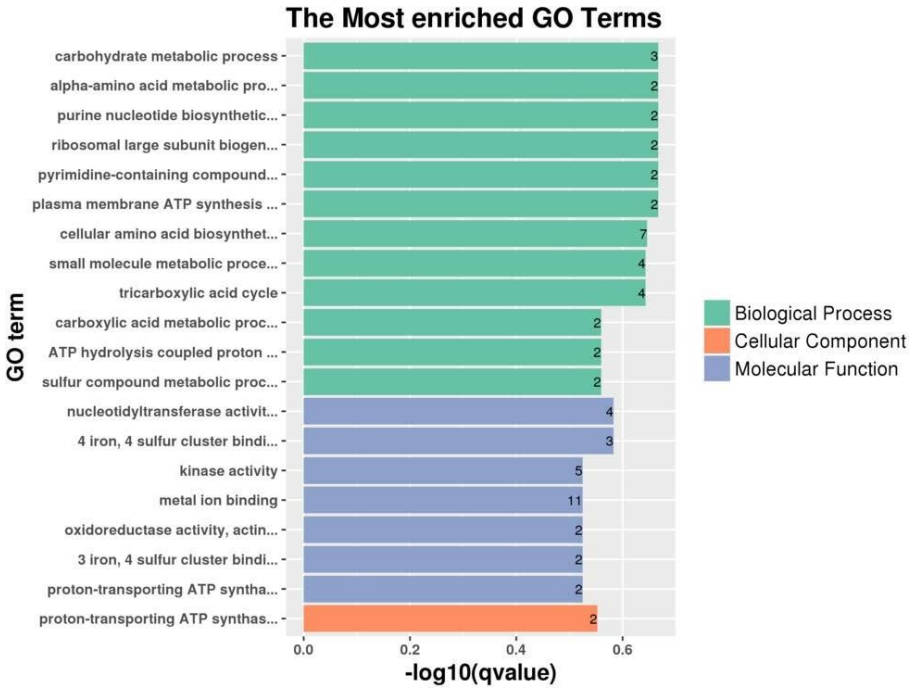
Appendix 50 - Statistics results of GO annotation genes for Cg (single 8h) vs. Cg (co-culture 8h) DEGs



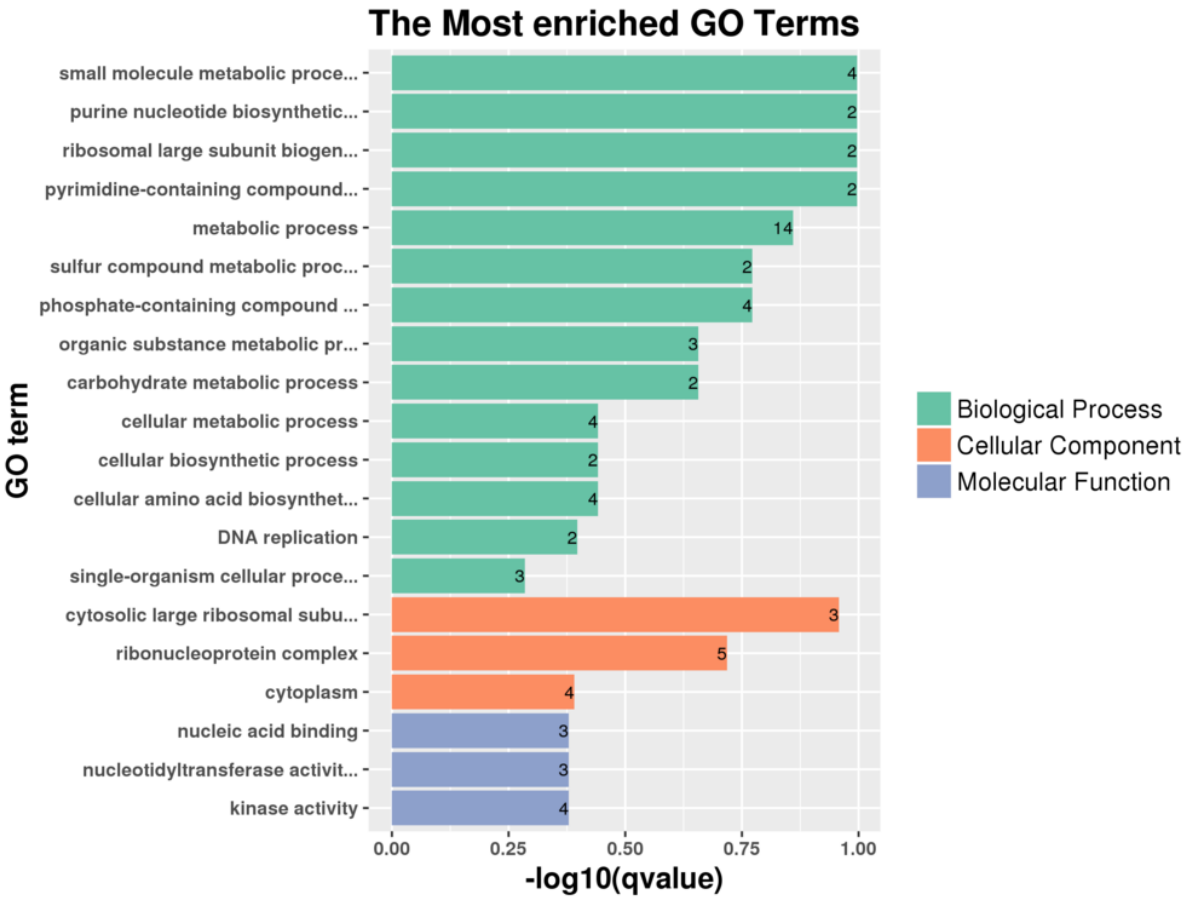
Appendix 51 - Statistics results of GO annotation for Lg (single 2h) vs. Lg (single 8h) DEGs



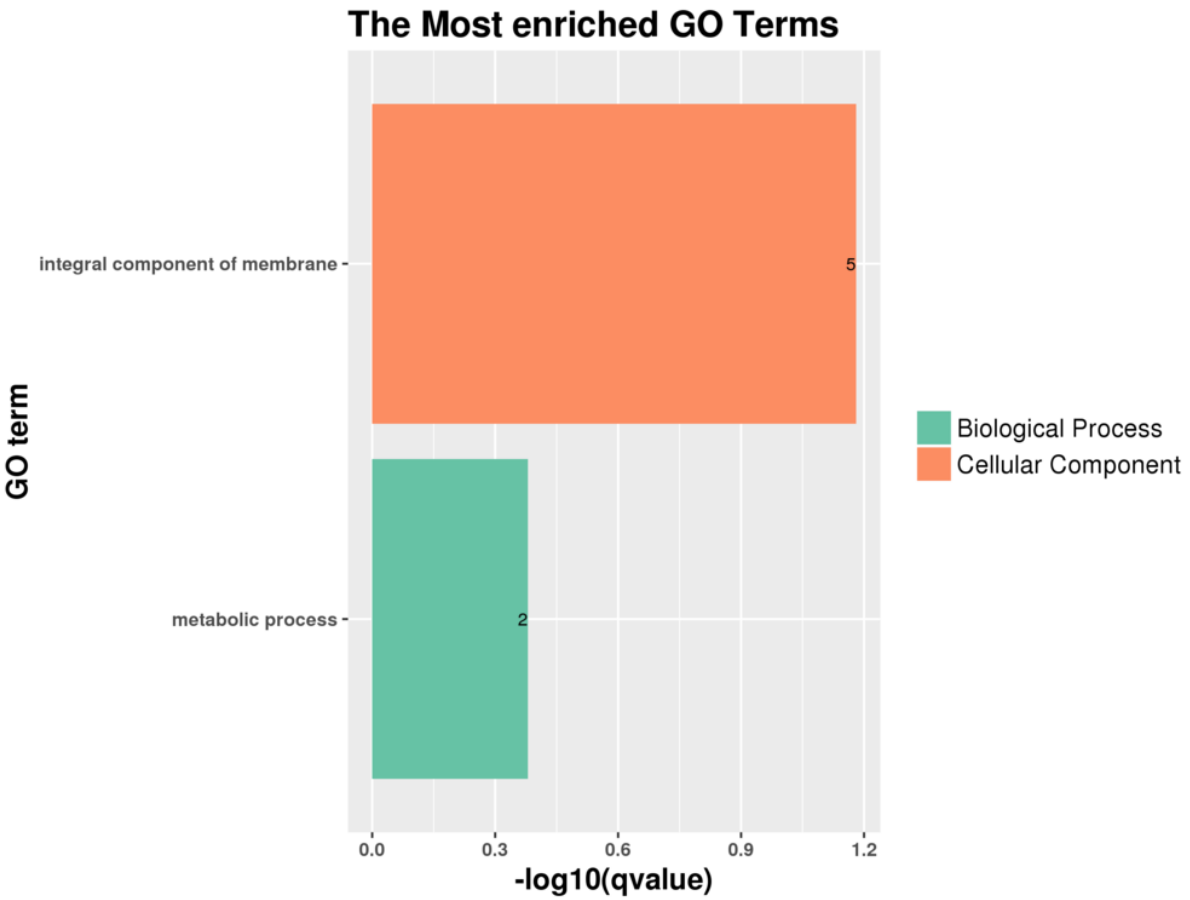
Appendix 52 - Statistics results of GO annotation for Lg (single 8h) vs. Lg (co-culture 8h) DEGs



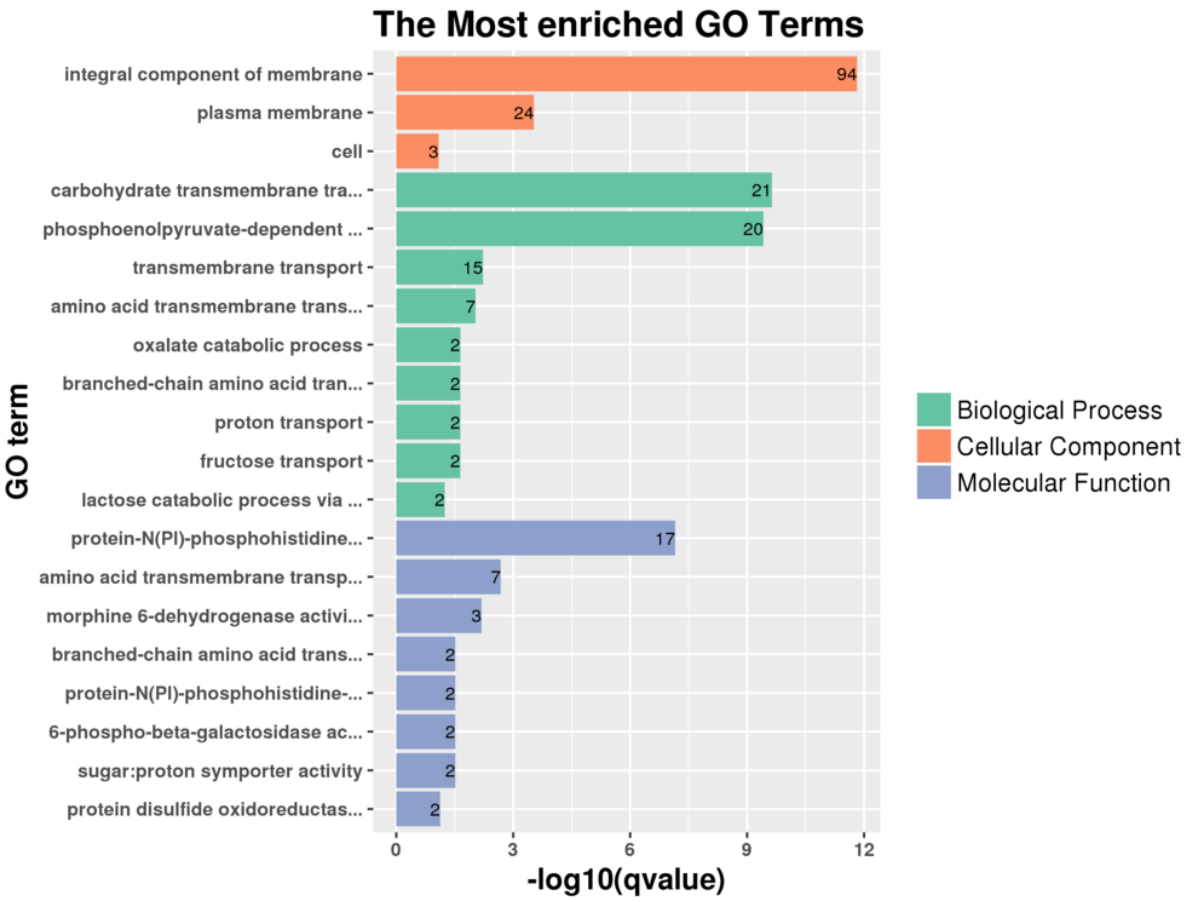
Appendix 53 - Bar plot of enriched GO term of differentially expressed genes for Cg (single 2h) vs. Cg (single 8h). The ordinate is the enriched GO term, the abscissa is the q-value of the term enrichment, and the number of differentially expressed genes is shown on the column. Different colors are used to distinguish biological process, cellular component and molecular function.



Appendix 54 - Bar plot of enriched GO term of differentially expressed genes for Cg (single 8h) vs. Cg (co-culture 8h). The ordinate is the enriched GO term, the abscissa is the q-value of the term enrichment, and the number of differentially expressed genes is shown on the column. Different colors are used to distinguish biological process, cellular component and molecular function.



Appendix 55 - Bar plot of enriched GO term of differentially expressed genes of *L. gasseri* for Lg (single 2h) vs Lg (single 8h). The ordinate is the enriched GO term, the abscissa is the q-value of the term enrichment, and the number of differentially expressed genes is shown on the column. Different colors are used to distinguish biological process, cellular component and molecular function.



Appendix 56 - Bar plot of enriched GO term of differentially expressed genes of *L. gasseri* for Lg (single 8h) vs. Lg (co-culture 8h). The ordinate is the enriched GO term, the abscissa is the q-value of the term enrichment, and the number of differentially expressed genes is shown on the column. Different colors are used to distinguish biological process, cellular component and molecular function.