TÉCNICO LISBOA

Masters in Biotechnology

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

Nuno Alexandre Aparício Pedro

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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.

Introduction

Vulvovaginal candidiasis (VVC) is the infection of the vulva and/or the vagina caused by species of the *Candida* genus. It is the second most common cause of vaginal infections[1] being estimated to affect 70-75% of women at least once in their lifetime, 40-50% of them experiencing at least one recurrence[2]. Among *Candida* spp., *C. albicans* is the more common causative agent of invasive and superficial fungal infections; however, in the recent years the number of infections caused by non-albicans *Candida* species (NCAC) has been raising significantly[3]. *C. glabrata* is now the second major cause worldwide of invasive fungal infections[4]. 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[5]) and also the presence of a competing microbiota[6]. Lactobacilii are among those species more often found to co-colonize niches with *Candida* spp being present at least in the gastrointestinal and urogenital tracts[7]. In fact, in the vaginal tract the microbiota has been found to be dominated by *Lactobacillus* species[8] out of which *L. acidophilus*, *L. crispatus*, *L. jensenii* and *L. gasseri* are usually the more abundant species[9]. . 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[10]. This thesis is focused in the effect exert 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 used to examine the ability of vaginal *C. glabrata* isolates or of a strain devoid of CgHAA1 gene, to grow in the presence of *L. jensenii* and *L. gasseri* was also examined. 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

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[11]	[12]
***C. albicans* SC5314**	Reference strain; clinical isolate recovered from the blood of an infected patient	-
***C. glabrata* Δhaa1**	KUE100_ΔCAGL0L09339g	[12]
***C. albicans* VG216**	Vaginal clinical isolate	[13]
***C. albicans* VG217**	Vaginal clinical isolate	[13]
***C. albicans* VG485**	Vaginal clinical isolate	[13]
***C. glabrata* BG2**	Vaginal clinical isolate	[13]
***C. glabrata* VG99**	Vaginal clinical isolate	[13]
***C. glabrata* VG281**	Vaginal clinical isolate	[13]
***C. glabrata* VG49**	Vaginal clinical isolate	[13]
***C. glabrata* VG216**	Vaginal clinical isolate	[13]
***L. gasseri* ATCC 33323**	Reference strain	DSMZ
***L. jensenii* ATCC 25258**	Reference strain	DSMZ

All the species were cultivated in MRS medium. 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).

Single-species or multi-species cultivation

To assess individual growth of *L. jensenii*, *L. gasseri* and all *Candida* strains in liquid MRS medium a pre-inoculum of each species was prepared by inoculating some colonies for 48h in liquid MRS, at 37ºC and using an agitation of 100 rpm. After 48h, 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 30ºC 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 48h 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, 0.2 or 0.4 for the two species 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.

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 100 μL 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.

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 □L 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.

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.

Effect of bacterial supernatant/or of bacterial cells 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 in 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 [14]. 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.

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.

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 was 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*

In order 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 1). 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.

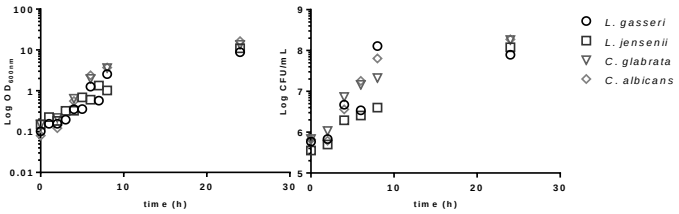

Figure 1 - Growth curve of C. glabrata, C. albicans, L. gasseri and L. jensenii in MRS medium at 37ºC with 100 rpm (top) and Log CFU of C. glabrata and C. albicans, L. gasseri and L. jensenii cultures in solid YPD and MRS, respectively (bottom).

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. As it could be seen the two *Candida* species are not able to grow on MRS plates supplemented with 32 and 64 mg/L fluconazole, 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. Similarly, the two Lactobacilli did not grow on MRS medium containing 300 mg/L tetracycline (, 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 (results not shown).

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

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. 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 2), while growth rate of the bacteria was inhibited by 52%, as detailed in Table 2. 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* (results not shown).

Table 2 – Growth rates (h^{-1}) of *C. albicans* and *L. gasseri* in co-culture with different proportions (yeast:bacteria) of *L. gasseri* at 37ºC, 100rpm and MRS medium

Culture	*L. gasseri* growth rate (h^{-1})	*C. albicans* growth rate (h^{-1})
***L. gasseri* single culture**	**0.25±0.05**	**----------**
C. albicans	**----------**	**0.35±0.09**
***C. albicans* + *L. gasseri* (1:1)**	**0.20**	**0.31**
***C. albicans* + *L. gasseri* (1:2)**	**0.12**	**0.31**
***C. albicans* + *L. gasseri* (1:4)**	**0.12±0.11**	**0.25 ±0.02**

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 3). 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% at 24h time point.

Table 3 – Growth rates (h^{-1}) of *C. albicans* and *L. jensenii* in co-culture with different proportions (yeast:bacteria) of *L. jensenii* at 37ºC, 100rpm and MRS medium

Culture	*L. jensenii* growth rate (h^{-1})	*C. albicans* growth rate (h^{-1})
***L. jensenii* single culture**	**0.17±4.38E-03**	**----------**
C. albicans	**----------**	**0.35±0.09**
***C. albicans* + *L. jensenii* (1:1)**	**0.16**	**0.37**
***C. albicans* + *L. jensenii* (1:2)**	**0.05**	**0.36**
***C. albicans* + *L. jensenii* (1:4)**	**0.06±2.62E-03**	**0.23±0.02**

Co-cultures between L. gasseri, L. jensenii and C. glabrata

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 Table 4. 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, comparing to the one obtained in single-culture (Table 4). 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 (results not shown). Notably, viability of *L. gasseri* cells was observed no major fluctuations at 24h (results not shown).

Table 4 – Growth rates (h^{-1}) of *C. glabrata* and *L. gasseri* in co-culture with different proportions (yeast:bacteria) of *L. gasseri* at 37ºC, 100rpm and MRS medium

Culture	*L. gasseri* growth rate (h^{-1})	*C. glabrata* growth rate (h^{-1})
***L. gasseri* single culture**	**0.25±0.05**	**----------**
C. glabrata	**----------**	**0.31±0.03**
***C. glabrata* + *L. gasseri* (1:1)**	**0.19**	**0.26**
***C. glabrata* + *L. gasseri* (1:2)**	**0.17**	**0.23**
***C. glabrata* + *L. gasseri* (1:4)**	**0.13±0.03**	**0.13±0.05**

The co-cultures with *L. jensenii* 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 5) was also reduced (about 57%) in the co-culture setting, comparing with the values registered in single-culture.

Table 5 - Growth rates (h^{-1}) of *C. glabrata* and *L. jensenii* in co-culture with different proportions (yeast:bacteria) of *L. jensenii* at 37ºC, 100rpm and MRS medium

Culture	*L. gasseri* growth rate (h^{-1})	*C. glabrata* growth rate (h^{-1})
***L. jensenii* single culture**	**0.17±4.38E-03**	**----------**
C. glabrata	**----------**	**0.31±0.03**
***C. glabrata* + *L. jensenii* (1:1)**	**0.18**	**0.20**
***C. glabrata* + *L. jensenii* (1:2)**	**0.06**	**0.17**
***C. glabrata* + *L. jensenii* (1:4)**	**0.06±0.03**	**0.13±0.05**

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

The results obtained showed a significant 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. A slight reduction in the growth rate was also clearly visible (Table 6). Consistent with these observations, the amount of ethanol present in the supernatant of the *C. glabrata* ∆haa1+*L. gasseri* co-culture was considerably lower the one registered in the supernatant of the *C. glabrata* ∆Haa1 single culture (results not shown). 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 place in co-culture with *C. glabrata* ∆haa1 compared with the wild type.

Table 6 - Growth rates of *C. glabrata* Haa1 and *L. gasseri* in co-culture with 1:4 proportion (yeast:bacteria) at 37ºC, 100rpm and MRS medium

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**

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

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 turn possible to assess how the genomic expression of *C. glabrata* would respond to the presence of lactobacilii, 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 4 and 8 h of inoculation. 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. 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 partially shown in the volcano plots shown in Figure 2. 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 remaining results are not shown.

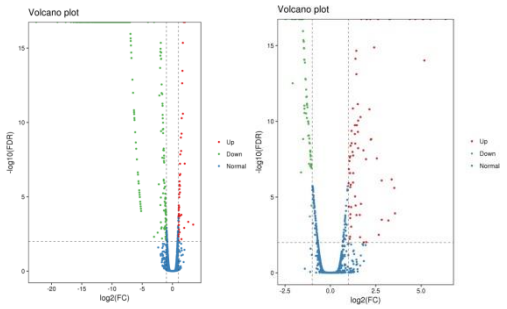

Figure 2 - Volcano plot (left) of differentially expressed genes for Cg (single 2h) vs. Cg (co-culture 2h); Volcano plot (right) of differentially expressed genes for Lg (single 2h) vs. Lg (co-culture 2h)

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

It was compared growth of a set of vaginal *C. glabrata* 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[13] and also the BG2 strain, a vaginal strain that had been studied before[13,15,16] 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* (Table 7). The extent of this reduction was similar (of about 30%) with exception of Vg485 that showed a higher decrease of growth rate (48%) comparing to the one observed in the laboratory strain SC5314 (28%).

Table 7 - 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

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%). 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% (results not shown). 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%) (Table 8). Showing that *C. glabrata* vaginal isolates are more adapted to the presence of *L. gasseri*.

Table 8 - Growth rates of *C. glabrata* vaginal isolates and *L. gasseri* in co-culture with 1:4 proportion (yeast:bacteria) at 37ºC, 100rpm and MRS medium

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**

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

The assays described until so far have explored co-cultures of Lactobacilii 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*[6,17–19], in which growth of the *Candida* spp is examined upon exposure to a supernatant obtained from a pre-culture of lactobacilii (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 (Figure 3). In the case of *C. albicans* inhibition is already observed when only 50 μL of the supernatant are used (Figure 3). It is important to stress that when only 100 μL 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 3).

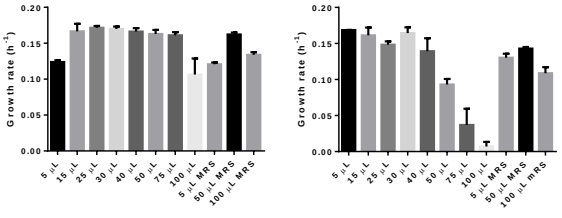

Figure 3 - Growth rate of *C. glabrata* and *C. albicans* with different concentrations of *L. gasseri* supernatant and MRS medium at 37ºC, 25 rpm in MMB medium. . The results shown were obtained from three independent experiments

Because biofilms are such a relevant virulence trait for *C. albicans* and *C. glabrata* cells[20–23], 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 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 4. 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 4. Nevertheless, the reduction induced by the supernatant was always more evident than the one attributable to the MRS medium Figure 4.

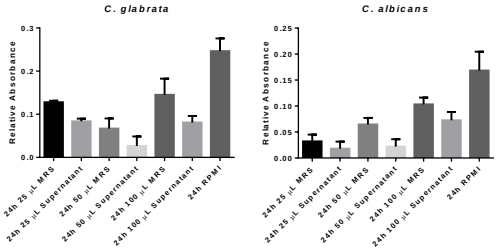

Figure 4 - Biofilm formation of *C. glabrata* and *C. albicans* at 24h in the presence of different concentration of *L. gasseri* supernatant and MRS medium at 37ºC, 25 rpm in RPMI medium. The results shown were obtained *from three independent experiments*

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, it was also examined how biofilm formation prompted by the two yeast species could be affected by the direct presence of Lactobacilii in the medium. 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, while *C. albicans* appeared as round shaped cells. Biofilm formation was observed for all the species tested. Co-cultivation resulted in the formation of a mixed biofilm comprising both *C. albicans* and Lactobacilii cells. It was possible to clearly observe the interaction between *L. gasseri* and *L .jensenii* and *C. albicans* cells. In all cases the increase of *Lactobacillus* species led to an increase of cell number, showing a much more packed biofilm. (Results not shown).

The effect of *L. gasseri* supernatant and of *L. gasseri* live cells in hyphae formation by *C. albicans* was also tested since this is another highly relevant virulence trait attributed to this yeast species[24,22,19]. 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. *C. albicans* cells cultivated in the absence of FBS showed no formation of hyphae in pH4 (results not shown). FBS supplementation induced, as expected, the formation of hyphae (results not shown), this being more evident at pH 7 than at pH 4 (results not shown). The presence of the bacterial supernatant reduced the number of visible hyphae (results not shown), 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 (results not shown).

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[25,26]. 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)[24]. 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[27]. 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[28]. 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*[19]. 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*[29,30]. 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 (results not shown) 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 (results not shown).

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[13,12], 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[12]. 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'[31]. *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[12]. 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[12]. 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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