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

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Abstract

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

Keywords: *Lactobacillus reuteri, C. albicans, C. glabrata, Lactobacillus – Candida* interference, supernatant.

Introduction

Throughout all their life, humans are associated with microorganisms whose presence has been increasingly claimed to contribute positively for human health (Thomas *et al.*, 2017; Goltsman *et al.*, 2018; Pascale *et al.*, 2018). The human intestine and the microbial communities that reside therein are a very good example of an excellently balanced mutualistic association (Martín *et al.*, 2014). In a healthy status, the intestinal microbiome has a balanced equilibrium between species. When dysbiosis occurs, microbiota turns into a polymicrobial community where didn't exist any predominant specie. This modification has been linked to an overgrowth of pathogens, including of Candida species, causing disease 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 (Fidel and Vazquez, 1999). *C. glabrata* is now the second major cause worldwide of invasive fungal infections (Castanheira *et al.*, 2014). Although little interest has been put on the commensal gut *Candida* populations, the more recent studies show that these populations can actually be responsible for triggering disseminated infections (Lopez-Medina and Y. Koh, 2016; Tong and Tang, 2017; Pappas *et al.*, 2018; Wheeler *et al.*, 2019). *C. albicans* penetrates the intestinal epithelium barrier by both fungal-induced endocytosis and active penetration, after which dissemination in the bloodstream occurs (Koh, 2013; Tong and Tang, 2017). The most important factors increasing dissemination of *C. albicans* from the gut are the lack of an effective host immune response, but an intestinal flora disorder has also been pinpointed as relevant (Prieto *et al.*, 2016; Tong and Tang, 2017; Pappas *et al.*, 2018; Witchley *et al.*, 2019)

In general, *Lactobacillus* have three main mechanisms to decrease of the pathogens' proliferation – the production of antimicrobial compounds (lactic acid, hydrogen peroxide, exopolysaccharides, bacteriocins and biosurfactants), found on the supernatant; by competition for adhesion sites and nutrients, and by modulating the host immune system (Boris and Barbés, 2000; Morales and Hogan, 2010). The most relevant of these metabolites is lactic acid which is largely produced by lactobacilli during the course of their metabolic activity (Jang *et al.*, 2019; Lourenço *et al.*, 2019). It has been shown that *Lactobacilli* species can produce exopolysaccharides that are able to exert a significant inhibitory effect against *Candida albicans*, not only inhibiting growth but also reducing filamentation and adhesion to vaginal epithelial cells (Badel, Bernardi and Michaud, 2011; Allonsius *et al.*, 2017).

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

Although some insights had been gathered concerning this inhibitory potential of Lactobacilli over pathogens (Candida species included), a thorough investigation on this interference mechanism has not been carried out, a knowledge that has the potential to contribute for the design of new therapeutic approaches by sensitizing Candida species through the improvement of Lactobacilli activity. Because interference of Lactobacilli over growth of pathogens has also been shown to occur in the gastrointestinal tract (Suhr and Hallen-Adams 2015), in this work we also examined how the intestinal species *L. reuteri*i interacts with *C. albicans* and *C. glabrata*, this being based on the identification of these two yeasts as part of the commensal gastrointestinal microbiota.

Materials and Methods

Strains and Media

The strains used in this work are listed in Table 1.

Table 1 - Description of the group of strains used in this study. The auxotrophy of *C. glabrata* ATCC2001 is his1trp2leu3.

Strain	Description	Source
C. glabrata KUE100	Laboratory strain derived from the reference CBS138	(Bernardo *et al.*, 2017)
C. albicans SC314	Laboratory strain	-
L. reuteri ATCC 23272	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 K2HPO4; 5 g/L Na-acetate; 2 g/L Ammonium oxalate; 0.20 g/L MgSO4 x 7H2O and 0.05 g/L MnSO4 x H2O. 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. reuteri* 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 (DO600nm) 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 DO600nm 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. reuteri* with *Candida* strains were performed using an experimental setup similar to the one described above for the single-species. In specific, a pre-inoculum of each species was left to grow for 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 DO600nm of 0.1, 0.2 or 0.4 for the species involved in the co-culture system. Growth of the 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 at 37ºC for 48, while the plates used to assess growth of *Candida* were put at 30C under aerobic conditions.

Effect of bacterial supernatant in growth of Candida

The supernatant of different hours of culture of L. The supernatants obtained along cultivation of *L. reuteri* in MRS medium was examined for its potential to inhibit growth of Candida in 200 uL-capacity 96-microwell plates For this, mid-exponential phase *C. albicans* and *C. glabrata* cells cultivated in MMB at pH4 were used to inoculate fresh this same medium supplemented with increasing amounts of the bacterial culture supernatants. The cell suspensions were prepared in 100 uL 2x concentrated MMB medium (at pH 4) to which was added the amount of the bacterial culture supernatant (5,15,25,30,40,50,75 and 100 µL) and sterile distilled water up to a volume of 200 µL. The microplates were incubated at 37ºC at an orbital agitation of 100 rpm. The OD600nm was measured after 2h, 4h, 6h 8h, and 24h of cultivation using a SPECTROstarNano from BMG LABTECH. The resulting growth curves were used to estimate the kinetic parameters.

Effect of lactic acid in growth of *Candida*

The effect of lactic acid on growth of *C. albicans* and *C. glabrata* was tested using as sources of the acid a mixture of the D and L isomers (DL-lactic

acid) or only L-lactic acid. 4g/L or 8g/L of lactic acid, from both sources, were used to supplement the MRS medium. For this, mid-exponential phase *C. albicans* and *C. glabrata* cells cultivated in MRS at pH4 were used to inoculate fresh this same medium supplemented with different concentrations of lactic acid. The microplates were incubated at 37ºC at an orbital agitation of 100 rpm. The OD600nm was measured after 2h, 4h, 6h 8h, and 24h of cultivation using a SPECTROstarNano from BMG LABTECH.

Extraction and purification of EPS obtained from *L. reuteri* cultures

For this, 10 mL of *L. reuteri* culture with an OD600nm of 0.6 were centrifuged at 15 000 g for 15 min at 4Cº. The pellet (EPS-b) resuspended in 1mL of PBS followed by a centrifugation at 11000g for 15 min. After that, the pellet was washed in 5 ml of sterile sodium chloride solution and then resuspended in 5 ml of 0.05 M EDTA to obtain the bound EPS fraction. The mixture was incubated under gentle agitation for 4 h at 4Cº and then centrifuged at 6000 g for 30 min at 4 ◦C. The EPS-b was precipitated from the supernatant by addition of two volumes of cold ethanol followed by an overnight incubation at 4Cº. After this time, the suspension was centrifuged during 30 min at 6000 g, at 4 ºC, and the EPS-b obtained was resuspended in 2 ml of distilled water. Afterwards this b-EPS was dialyzed (molecular weight cut-off: 6000–8000 Da) against 5L of distilled water for 2 days with three water changes per day. To obtain the EPS released into the culture broth, the supernatant resulting of the first centrifugation was used (EPS-r). Afterwards, 2mL of 20% trichloroacetic acid was added and the mixture was incubated for 2h at 4Cº under gentle agitation. The precipitated proteins were removed by centrifugation at 25 000 g for 20 min at 4 ºC and the EPS was precipitated from the supernatant using two volumes of cold ethanol followed by an overnight incubation at 4 ºC. After this time, the suspension was centrifuged at 6000 g for 30 min at 4Cº and the pellet containing EPS-r was resuspended in 2 ml of distilled water and dialyzed as described for the EPS-b fraction. The total amount of carbohydrates present in the EPS was determined using the phenol/sulfuric acid method and using glucose as a standard. The quantification of EPS was expressed in g/L, equivalent of glucose per liter of growth medium. The purified EPS-b and EPS-r fractions were used to supplement the MRS growth medium where *C. albicans* and *C. glabrata* were cultivated (0 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml. For this, mid-exponential phase *C. albicans* and *C. glabrata* cells cultivated in MMB at pH4 were used to inoculate fresh this same medium supplemented with increasing amounts of EPS. The cell suspensions were prepared in 100 □L 2x concentrated MMB medium (at pH 4) to which was added the amount of EPS and sterile distilled water up to a volume of 200 □L. The microplates were incubated at 37ºC at an orbital agitation of 100 rpm. The OD600nm was measured after 2h, 4h, 6h 8h, and 24h of cultivation using a SPECTROstarNano from BMG LABTECH.

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

The effect of a 48h *L. reuteri* culture supernatant or of the *L. reuteri* live cells on the ability of *C. albicans* cells to trigger filamentation was tested in: a) RPMI medium also inoculated with *L. reuteri* cells (OD=0,4) (before cultivated in MRS medium) in a proportion of 1:4 (yeast:bacteria); b) in RPMI supplemented with 50% bacterial culture supernatant; c) RPMI supplemented with 10% FBS (fetal bovine serum). All the observations were obtained with a Zeiss microscope using a 1000x magnification. After inoculation of the *C. albicans* cells at an OD600nm of 0.1 under the different conditions, samples of the cultures were taken for microscopic analysis. All the observations were obtained with a Zeiss microscope.

Effect of bacterial supernatant/or of bacterial cells in *C. glabrata* and *C. albicans* biofilm formation.

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

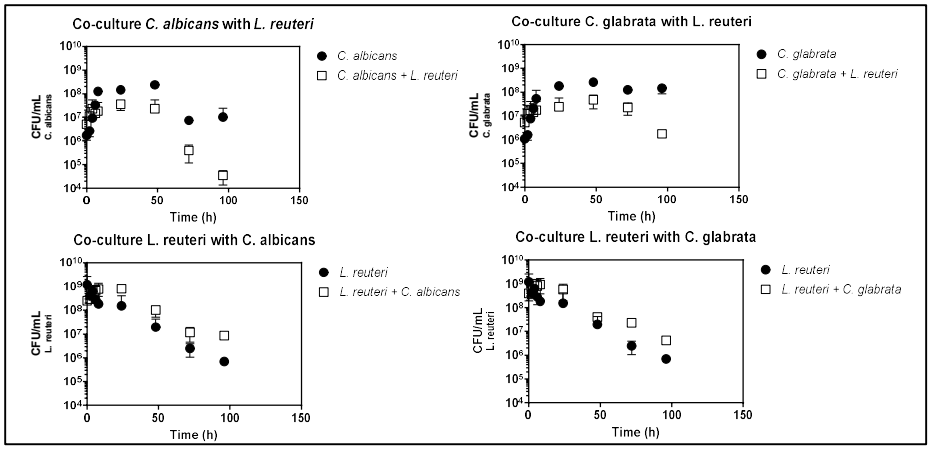

Figure 1 - Growth (CFU/mL) of the co-culture between *C. albicans*/*C. glabrata* with *L. reuteri*. The *L. reuteri* growth was determined based on the number of CFUs obtained on MRS plates supplemented with 96 mg/L fluconazole. The *Candida* growth was determined based on the number of CFUs obtained on YPD plates supplemented with 300 mg/L tetracycline. The results shown were obtained from three independent experiments.

Results

Co-cultures between *L. reuteri* and *C. albicans* /*C. glabrata*

Growth of *L. reuteri*, *C. albicans* and *C. glabrata* in single and in co-culture was followed in MRS medium during 96h and the result obtained are shown in

Figure 1. The first observation that comes out of these results is the loss of viability that is observed in the *L. reuteri* population either in single and co-culture, this being more pronounced in the single-culture (in line with the also observed "protective" effect exerted by the presence of the two Candida species). This was also accompanied by a reduction in the viability of the two Candida species, particularly in the case of *C. albicans* that suffered a very prominent reduction in viability (around 43,4%-fold reduction, compared to the maximum number of viable cells achieved in the co-culture). The fact that the reduction in viability is observed for the later periods of cultivation suggests the accumulation of one or more inhibitory products in the supernatant that, in the end, contribute to reduce the viability of the Candida species.

L. reuteri supernatant through time

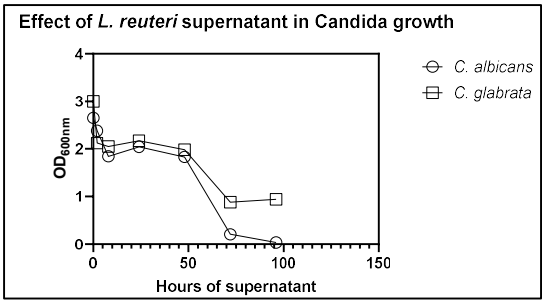

Figure 2 - Growth curve of *C. albicans* and *C. glabrata* with *L. reuteri* supernatant harvested in different times (0h, 2h, 4h, 8h, 48h, 72h, 96h) in MRS medium at pH 4 at 37ºC with 100 rpm.

Based on this results that show reduced viability of *C. albicans* and *C. glabrata* in the later stages of the co-culture we decided to examine whether a similar inhibitory effect with *L. reuteri* supernatant. The Figure 2 confirmed that this is the case with the supernatants obtained at later stages of *L. reuteri* cultivation being much more inhibitory than those obtained at earlier stages. As conclusion, there is evidence exists something in the supernatant that accumulates over time, that is responsible for the inhibition of Candida (Figure 2). With this, it was subsequently analyzed whether this antimicrobial compound was a metabolite of Lactobacillus. for example, lactic acid (LA).

The influence of lactic acid on yeasts growth

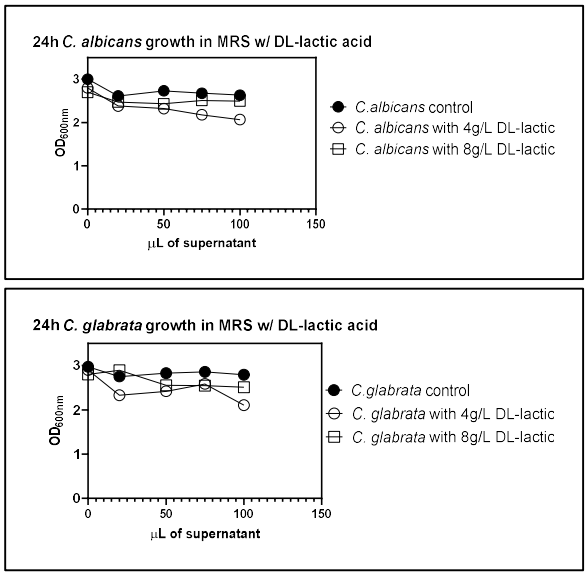

Figure 3 - Growth curve of *C. albicans* and *C. glabrata* in MRS medium with 4g/L with 8g/L of DL-lactic acid.

Under the conditions we have used to cultivate *L. reuteri* the maximum amount of lactic acid present in the supernatants was of 8 g/L which, as shown in Figure 3 has no significant inhibitory effect over growth of *C. albicans* or *C. glabrata*. As predicted, and previously found in this laboratory, the presence of lactic acid (regardless of concentration) does not influence yeast growth (Lourenço *et al.*, 2019). Through these results, it should be concluded that not is the LA responsible for inhibition, having no effect on *Candida* growth.

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

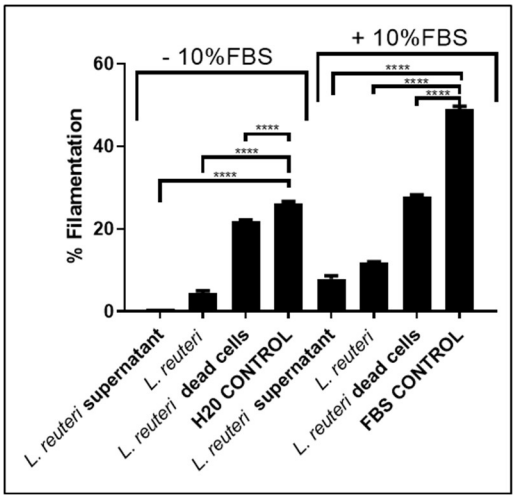

Figure 4 - Filamentation of *C. albicans* after 24h in the presence of different concentration of *L. reuteri* supernatant, *L. reuteri* live cells and *L. reuteri* dead cells. The experiment was performed under and MRS medium at 37ºC, 25 rpm in RPMI medium. The results shown were obtained from two independent experiments.

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

when the *L. reuteri* cells are killed before inoculation in the RPMI medium (Figure 4).

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

The ability to produce biofilm is another important virulence factor of Candida cells (Silva *et al.*, 2009) and the ability to form mixed biofilms with lactobacilli species, including *L. gasseri*, has been reported (Pedro, 2017; Tan *et al.*, 2017; Matsuda *et al.*, 2018; Rossoni *et al.*, 2018). As such, in this work we have examined how the presence of *L. reuteri* would influence the ability of *C. albicans* and *C. glabrata* to form biofilms on the surface of polystyrene. The results obtained (Figure 5 and Figure 6) show the ability of these cells to form tight mixed-biofilms being observed the establishment of tight interactions among them (Tan *et al.*, 2017).

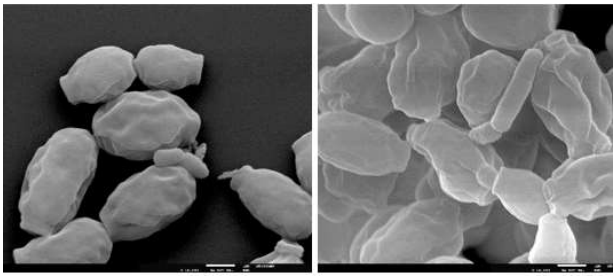

Figure 5 - Co-culture of *L. reuteri* with *C. albicans* from 0.1 initial OD culture observed by SEM with a magnification of x10000.

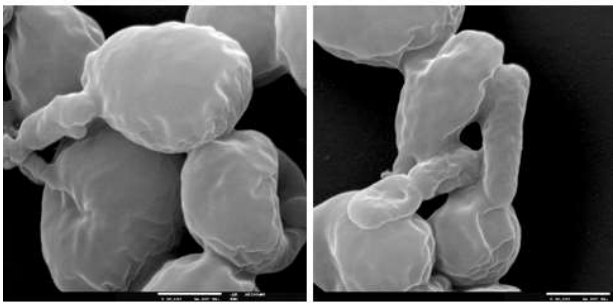

Figure 6 - Co-culture of *L. reuteri* with *C. glabrata* from 0.1 initial OD culture observed by SEM with a magnification of x20000.

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

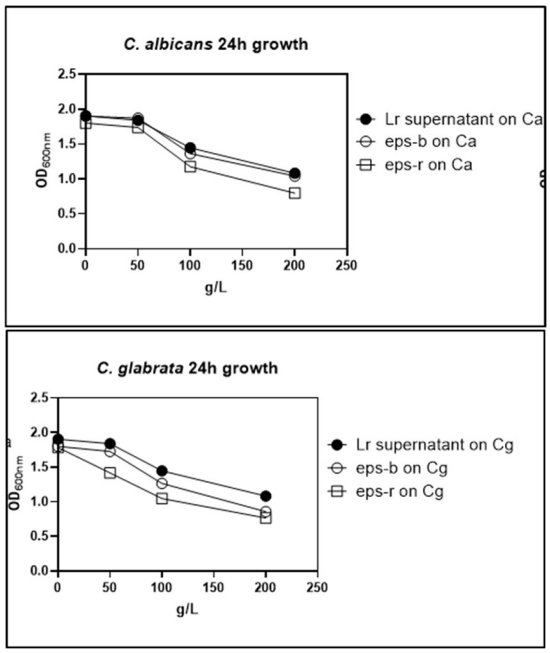

Figure 7 - Growth curve of *C. albicans* and *C. glabrata* with different concentrations of EPS-b and EPS-r from *L. reuteri* supernatant.

Since the exopolysaccharide of other lactobacilli species has been shown to contribute for inhibition of growth and virulence of Candida (Badel, Bernardi and Michaud, 2011; Sungur *et al.*, 2017), in this thesis we have examined whether this would also be detected for the exopolysaccharides produced by *L. reuteri*. For this, we have measured the effect of the exopolysaccharide bound to the bacterial cell (EPS-b) or of the one released (EPS-r) to the broth. After purification of these fractions, their effect in inhibition of *C. albicans* and *C. glabrata* growth was tested. Cultivation of *L. reuteri* in 10 mL of the MRS medium led to the isolation of 0.254g/L of EPS-b and 0.439g/L of EPS-r. Both the EPS-b and EPS-r showed ability to inhibit growth of *C. albicans* and *C. glabrata*, with the EPS-r being slightly more inhibitory (Figure 7).

Discussion

The results obtained do not support the idea that lactic acid is the inhibitory product underlying the observed toxic effect since concentrations identical to those present in the bacterial supernatant did not produce a significant inhibitory effect on growth of the two yeast species. In previous studies, the independence of yeast growth had been proven in the presence or absence of lactic acid (Lourenço *et al.*, 2019), suggesting there is other acid molecules could be present and could mediate this effect, disregarding lactic acid. A more thorough metabolomic characterization of this bacterial supernatant and how its composition changes along the cultivation time of *L. reuteri* in MRS has to be performed to better clarify this. Thus, it can be interesting to study the lactobacillus secretome in order to analyze the proteins present in the supernatant in the absence and presence of *Candida*.

In this paper it is demonstrated that this intestinal species has a strong potential to inhibit growth of *C. albicans* and *C. glabrata*. This inhibitory effect may be due the production of EPS, which have already been shown to inhibit inflammation factors released by pathogens and decreased its adherence (Matsubara *et al.*, 2016). Although several studies have already reported EPS effects on *Candida* from other Lactobacillus species (Allonsius *et al.*, 2017; Jørgensen *et al.*, 2017) it would be interesting to infer the real inhibiting role of EPS, exploring the genes involved in the production of EPS and analyzing if there is any altered expression in that genes when in co-culture.

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

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