Influence of glucose concentration on the structure and quantity of biofilms formed by *Candida parapsilosis*

Leonel Pereira[1], Sónia Silva[1*], Bruno Ribeiro[1], Mariana Henriques[1], Joana Azeredo[1]

[1] CEB, Centre of Biological Engineering, LIBRO – Laboratório de investigação em Biofilmes Rosário Oliveira, University of Minho, Campus de Gualtar 4710-057, Braga, Portugal

Corresponding author: *Centre of Molecular and Environmental Biology, Universidade do Minho, Campus de Gualtar 4710-057, Braga, Portugal. Phone: +351 53 604 419; Fax: +351 253678986; E-mail: soniasilva@deb.uminho.pt

Running title: Glucose *Candida parapsilosis biofilm modulation*

Abbreviations: Actin (*ACT1)*; American Type Culture Collection (ATCC); cell wall regulator 1 (*BCR1*); Colony-forming units (CFUs); crystal violet (CV); glucan synthase (*FKS1*); non-transcriptase reverse controls (NRT)**;** Sabouraud dextrose agar (SDA); Sabouraud dextrose broth (SDB); stearoyl-coenzyme A (CoA) desaturase 1 (*OLE1*); scanning electron microscopy (SEM)

SUMMARY

Candida parapsilosis is nowadays an emerging opportunistic pathogen and its increasing incidence is part related to the capacity to produce biofilm. In addition, one of the most important *C. parapsilosis* pathogenic risk factors includes the organisms' selective growth capabilities in hyper alimentation solutions. Thus, in this study, we investigated the role of glucose in *C. parapsilosis* biofilm modulation, by studying biofilm formation, matrix composition and structure. Moreover, the expression of biofilm-related genes (*BCR1*, *FKS1* and *OLE1*) were analyzed in the presence of different glucose percentages. The results demonstrated the importance of glucose in the modulation of *C. parapsilosis* biofilm. The concentration of glucose had direct implications on the *C. parapsilosis* transition of yeast cells to pseudohyphae. Additionally, it was demonstrated

that biofilm related genes *BCR1*, *FKS1* and *OLE1* are involved in biofilm modulation by glucose. The mechanism by which glucose enhances biofilm formation is not fully understood, however with this study we were able to demonstrate that *C. parapsilosis* respond to stress conditions caused by elevated levels of glucose by up-regulating genes related to biofilm formation (*BCR1*, *FKS1* and *OLE1*).

KEYWORDS: *Candida parapsilosis*; glucose; biofilm; virulence genes

INTRODUCTION

Candida species are an increasingly common cause of oral infections (Williams *et al.*, 2011). Although *Candida albicans* is still the most commonly isolated, other *Candida* species such as *Candida parapsilosis* are increasingly reported (Martins *et al.*, 2010; Moris *et al.*, 2012). This rise in incidence is at least in part related to the organism's ability to produce biofilms on medical devices (Al-Fattani & Douglas, 2006; Ramage *et al.*, 2006; Silva *et al.*, 2009b). As it is known, biofilm is a community of microbes attached to a surface and embedded in an extracellular matrix (Al-Fattani & Douglas, 2006; Ramage *et al.*, 2006). Moreover, the most problematic characteristic of biofilms is their higher increased tolerance to common antifungal agents comparatively to their planktonic counterparts (Taff *et al.*, 2013). *Candida* biofilm formation and matrix composition are strongly dependent on the environmental factors, such as salivary flow, pH and carbohydrates diet (Akpan & Morgan, 2002). In this respect, the exposure to different quantities of glucose influences candida biofilm formation ability and composition. For example, *C. albicans* increases the ability to produce extracellular and intracellular polysaccharides in response to high glucose levels, which modulates biofilm development and matrix composition (Santana *et al.*, 2013). We have previously demonstrated that *C. parapsilosis* biofilms consist of a dense network of yeast and/ or pseudohyphae with large amounts of carbohydrates (Silva *et al.*, 2009a). One of most important risk factors of the *C. parapsilosis* strains included the organism's selective growth capabilities in hyper alimentation solutions (Almirante *et al.*, 2006). Indeed, it has been demonstrated that *C. parapsilosis* can proliferate in high concentrations of glucose (Shin et al., 2002), however, it remains unclear if the presence of glucose could affect the biofilm development and, matrix composition as a consequence of the up-regulation of genes involved in biofilm formation and maturation such as cell wall regulator 1 (*BCR1*), glucan synthase (*FKS1*) and stearoyl-coenzyme A (CoA) desaturase 1 (*OLE1*) (Nguyen *et al.*, 2011b; Taff *et al.*, 2012; Pannanusorn *et al.*, 2013).

Thus, this work aimed at studying the influence of glucose in biofilm modulation by *C. parapsilosis* strains. Specifically, elucidating the involvement of *BCR1*, *FKS1* and *OLE1* genes in *C. parapsilosis* biofilm development, structure and matrix composition, in the presence of glucose.

MATERIAL AND METHODS

Organisms

Two *C. parapsilosis*, one oral isolate, *C. parapsilosis* AD, from the collection of the Biofilm Group of the Centre of Biological Engineering, University of Minho (Braga, Portugal) and originally obtained from the Clinical of Dentistry, Congregados (Braga, Portugal); and a reference strain of *C. parapsilosis* (ATCC 22019) from the American Type Culture Collection (Manassas, VA, USA) were used in this work. The identity of the clinical isolate was initially confirmed by PCR-based sequencing using specific primers (ITS1 and ITS4) that are specific for the 5.8 subunit gene (Williams *et al.*, 1995).

Growth conditions

For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 48 h at 37 °C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck) and incubated for 18 h at 37 °C under agitation at 120 rev min^{-1}. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4 °C and washed twice with phosphate-buffered saline (PBS, pH 7). The yeasts were then enumerated using an improved Neubaeur haemocytometer and adjusted to a concentration of 1×10^7 cells ml^{-1}. The cell pellets were finally ressuspended in RPMI 1640 (pH 7; Sigma Saint Louis, Missouri, USA) with increase percentages of the glucose, 0.2, 2 and 10 % (w/v).

Biofilm formation

Biofilms were formed in 96-wells microtiter plates (orange Scientific, Braine-l´Alleud, Belgium). For that, standardized inoculum (200 µl of yeast cell suspension containing 1×10^7 cells ml^{-1} in RPMI with 0.2, 2 and 10 % of glucose w/v) were placed into selected wells and incubated at 37 °C in an orbital shaker incubator at 120 rev min^{-1} during 24 h.

Biofilm characterization

Total biomass quantification

Biofilm formation ability was assessed through quantification of total biomass by crystal violet (CV) staining (Silva *et al.*, 2009a). After biofilm formation, the culture medium was aspirated and the non-adherent cells were removed by washing the biofilms once with PBS. Then, the biofilms were fixed with 200 µl of methanol, which was removed after 15 min of contact. The microtiter plates were allowed to dry at room temperature, and 200 µl of CV (1 % v/v) were added to each well and incubated for 5 min. The wells were then gently washed twice with sterile ultra-pure water and 200 µl of acetic acid (33 % v/v) added to release and dissolve the stain. The absorbance of the solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570 nm. Experiments were repeated as part of three to five independent assays. The results were presented as A_{570} values cm^{-2}. The strains were classified as low biofilm-forming, moderate biofilm-forming, and higher biofilm- forming according to their cut-offs by CV (<0.44; 0.44-1.17, and >1.17) (Zambrano et al., 2014).

Quantification of cultivable cells

The number of cultivable cells was determined by counting colony-forming units (CFUs) following biofilm cells ressuspension (Silva *et al.*, 2010). Briefly, biofilms were first washed once with PBS to remove loosely attached cells and the biofilm was then ressuspended by repeated pipetting. The biofilms were removed by scrapping with the help of pipet tips and the complete removal was confirmed by subsequent CV staining and spectrophotometric reading for inspection of the wells. The ressuspended biofilm (500 µl) was vigorously vortexed for 5 min to disrupt the biofilm matrices and then serial decimal dilutions (in PBS) were plated onto SDA. Agar plates were incubated for 24 h at 37 °C, and the total CFUs per unit area [Log (CFUs cm^{-2})] of microtiter plate well were enumerated. Experiments were repeated on three occasions with individual samples evaluated in triplicate.

Biofilm structure

The structure of biofilms was examined by scanning electron microscopy (SEM). For that, 2 ml of each standardized suspension (1×10^7 cells ml^{-1} in RPMI with 0.2, 2 and 10 % of glucose) was incubated into 24-well microtiter plates (orange Scientific, Braine-l´Alleud, Belgium) at 37 °C and 120 rev min^{-1}. After 24 h, the medium was aspirated and non-adherent cells removed by washing the biofilm once with sterile ultra-pure water. Samples were dehydrated with alcohol (using 70 % ethanol for 10 min, 95 % ethanol for 10 min and 100 % ethanol for 20 min) and air dried for 20 min. Samples were kept in a

desiccator until the bases of the wells were removed using the burning scalped. Prior to observation, the base of the wells was mounted onto aluminium stubs, support coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

Biofilm matrix composition

Extraction method and quantification

Biofilms were formed also in 24-well microtiter plates (orange Scientific, Braine-l´Alleud, Belgium) for 24 h at 37 °C and 120 rev min-1 as previously described. After 24 h of biofilm formation the matrix was extracted using a slight modification to previously described protocols (Azeredo et al., 2003; Silva et al., 2009a). Briefly, biofilm samples were scraped from the plates, ressupended with ultra-pure water, sonicated (Utrasonic Processor, Cole-Parmer, IIlinois, USA) for 30 s at 30 W, and then the suspension was vortex for 2 min. Then, 1 ml of each suspension was filtered in a whatman membrane filter 0.45 µm and allowed to dry at 60 °C until constant dry biofilm weight was determined.

Total carbohydrate and β-1,3 glucan quantification

Total carbohydrate content of the biofilm matrix was estimated by the Phenol-sulfuric acid method according to the procedure of (DuBois et al., 1956), using glucose as standard. For that, 500 µl of each sample was to mix with 500 µl of phenol (50g/l) and 2.5 ml of sulfuric acid (95%-97%). The results were presented as the mg of carbohydrate per g of biofilm dry weight.

Matrix β-1,3 Glucan measurements

β-1,3 glucan content in the matrix was determined using the limulus lysate assay Glucatell® (1,3)-b-D-glucan detection reagent kit (Associates of Cape Cod, MA), according the manufacturer's instructions and as previously described (Taff *et al*, 2012). The quantity of β-1,3 glucan was measured at 405 nm and was normalized by the total dry weight of carbohydrates and presented as the ng of β-1,3 glucan per mg of carbohydrate.

Gene expression analysis

Primers for real time-PCR were designed using Primer3 web-based software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and are listed in Table 1. In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were firstly amplified from *C. parapsilosis* strains genomic DNA.

Biofilms cells RNA extraction

For the molecular approach, biofilms of *C. parapsilosis* strains were grown in 24-wells microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) in three different conditions, 0.2, 2 and 10 % of glucose in RPMI, with a final concentration of 1×10^{7} cells ml^{-1} during 24 h (as described above). After, biofilm formation the medium was aspired and the wells were washed once with ultra-pure water to remove non-adherent cells. Then, the biofilms were scraped from wells with 1 ml of PBS and sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W to separate the cells from the biofilm matrix. The cells were harvested by centrifugation at 3000 *g* for 10 min at 4 °C.

RNA extraction

RNA extraction was performed using PureLink® RNA Mini Kit (Invitrogen, Carlsbad, USA). Prior to RNA extraction, a lysis buffer (PureLink® RNA Mini kit) was prepared adding 1 % of *ß*-mercaptoethanol. Then, 500 µl of lysis buffer and glass beads (0.5 mm diameter) were added to each pellet. These mixes were homogenized twice for 30 s using a Mini-Bead-Beater-8 (Stratech Scientific, Soham, UK). After cells disruption, the PureLink® RNA Mini Kit (Invitrogen, Carlsbad, USA) was used for total RNA extraction according to the manufacturer's recommended protocol. To avoid potential DNA contamination samples were treated with RNase-Free DNase I (Invitrogen, Carlsbad, USA). The RNA extraction was performed at three different independents assays.

Synthesis of cDNA

To synthesize the complementary DNA (cDNA) the iScript cDNA Synthesis Kit (Bio-Rad, Berkeley, USA) was used according to the manufacturer's instructions. For each sample, 0.5 µg of the extracted RNA was used for cDNA synthesis. cDNA synthesis was

performed firstly to 70 °C for 5 min and then 42 °C for 1 h. The reaction was stopped by heating for 5 min at 95 °C.

Quantitative Real-Time PCR (qRT-PCR)

Real-time PCR (CF X96 Real-Time PCR System; Bio-Rad, Berkeley, USA) was used to determine the relative expression levels of *BCR1*, *FKS1* and *OLE1* genes concerning *C. parapsilosis* strains. *ACT1* gene for *C. parapsilosis* was used as housekeeping gene (Silva *et al.*, 2009b). Each reaction mixture consisted of a working concentration of SsoFast EvaGreen Supermix (Bio-Rad, Berkeley, USA), 50 µM forward and reverse primers, and 4 µl cDNA, in a final reaction volume of 20 µl. Negative controls, as well as, non-transcriptase reverse controls (NRT) were included in each run. The relative quantification of genes expression was performed by the Pfaffl method (Pfaffl, 2004) and normalized to levels of an *ACT1* internal control gene as follows: ration = $(E_{target})^{\Delta CTtarget(control\text{-}treated)}/(E_{ACT1})^{\Delta CTACT1(control\text{-}treated)}$. Each reaction was performed in triplicate and mean values of relative expression were determined for each gene.

Statistical Analysis

Results were compared using a two-way ANOVA with the Bonferroni test, using GraphPad Prism 5 software. All tests were performed with a confidence level of 95 %.**RESULTS**

Influence of glucose on biofilm formation

Biofilms formed in the presence of different concentrations of glucose were analyzed by complementary methodologies, total biomass using CV staining [Abs cm^{-2}] (Fig. 1a) and quantification of cultivable cells [Log (CFUs cm^{-2})] (Fig. 1b). It was evident that both strains were able to form biofilm, although in different extents depending on the strain and amount of glucose. *C. parapsilosis* ATCC 22019 could be considered as a low biofilm-forming strain (CV <0.44) and *C. parapsilosis* AD as a high biofilm-forming strain (CV >1.1), with the *C. parapsilosis* clinical isolate presented 3 times more biomass than the reference strain ($p<0.0001$).

In general, glucose enhanced biofilm formation by both strains, being more evident for the clinical isolate which is a strong biofilm producer, with values of CV statistically higher ($p<0.0001$) at 10 % glucose, when compared to the lower concentrations.

The biofilms were also analyzed in terms of total amount of cultivable cells [Log (CFUs cm^{-2})] (Fig. 1b) and likewise CV results the number of cells increased with the increase in glucose concentration and the number of viable cells of biofilms formed by strain *C. parapsilosis* AD was significantly higher ($p<0.0001$) at 10 % glucose, when compared to the lower concentrations.

Influence of glucose on biofilm structure

In order to understand the role of glucose on biofilm ultrastructure and *C. parapsilosis* cells morphology, biofilms formed under different concentrations of glucose were observed by SEM (Fig. 2).

Mature biofilms of *C. parapsilosis* presented different structures and cells exhibited different morphological characteristics depending on the strain and glucose concentration. Curiously, it was possible to observe that the two strains presented completely different biofilm structures. *Candida parapsilosis* ATCC 20019 biofilm, the weaker biofilm producer, consisted of aggregates of yeasts that increased in thickness with the increase of the glucose. It is important to emphasize that the oral isolate *C. parapsilosis* AD, the stronger biofilm former, despite presenting a biofilm composed by non-contiguous aggregates of cells, the cells switched from yeasts to pseudohyphae cells with the increased of glucose concentration (see arrow in Fig. 2).

Influence of glucose on carbohydrate matrix content

The Dubois method and Glucatell® Kit were used to determine total carbohydrates and specifically β-1,3 glucan contents in the matrices of biofilms formed in different concentrations of glucose (Table 2).

The biofilms matrix compositions were dependent on the presence of glucose in the biofilms growth media. The results showed that, *C. parapsilosis* biofilm had high amounts of matrix, total carbohydrates and β-1,3 glucan with increased glucose concentration. In addition, the values of carbohydrates increased for ATCC 20019, but slightly decreased for *C. parapsilosis* AD between 2 and 10%. Moreover, the values of β,1,3 glucans were in accordance with the quantity of total carbohydrates identified with a stronger increase for 0.2% to 2% and 10% of the glucose for both strains. It is

important to stress that *C. parapsilosis* biofilms grown in the presence of 0.2 % of glucose the amount of carbohydrates and β-1,3 glucans were below the detection limits.

Influence of glucose in *BCR1*, *FKS1* and *OLE1* genes expression

In order to understand how glucose modulates biofilm composition and structure, we assessed the expression levels of genes involved in the production of matrix components. Specifically we studied the relative expression levels of *FKS1* gene, which codifies a cell-membrane enzyme that produces β-1,3 glucan, from glucose, that is further incorporated into the biofilm matrix; the *BCR1* which codifies a regulator protein important for encoding biofilm matrix proteins; and the *OLE1* gene that codes for the stearoyl-CoA desaturase that plays essential roles in lipid synthesis. The *ACT1* gene, that is constitutively expressed, was selected as the housekeeping gene. Fig. 3 presents the gene expression levels of *BCR1*, *FKS1* and *OLE1* of biofilm cells grown in 0.2, 2 and 10 % of glucose.

Both strains of the *C. parapsilosis*, ATCC 20019 and AD¸ showed a tendency to increase the levels of expression of the three genes with the increase of glucose. In fact, biofilms of *C. parapsilosis* ATCC 20019 exhibited an up-regulation of approximately 1.5 fold of *BCR1*, more than 5 fold of *FKS1* and 4 fold of *OLE1* in the presence of high amounts of glucose. The relative expression of the 3 genes in the oral isolate *C. parapsilosis* AD presented also an up-regulation with the increase of glucose, in which it was observed a high increase at 10 % of glucose (almost 4 and 10 folds in *FKS1* and *OLE1* genes, respectively).

DISCUSSION

The success of *C. paraspilosis* colonization is mostly due to their ability to form biofilms. The human oral mucosa is exposed to different concentrations of sugars that can modulate biofilm formation. In fact, glucose plays an important role as energy and carbon source in *Candida* genus. In this sense, it is important to disclose how *C. parapsilosis* is able to adapt to different percentages of glucose and thus understand the increased number of candidiasis due to *C. parapsilosis* biofilms (Trofa *et al.*, 2008). In this work we investigated the composition and structure of biofilms formed by two strains

of *C. parapsilosis*, a collection strain and an oral isolate. Furthermore we studied the levels of expression of genes known to be enrolled in biofilm formation.

The presence of glucose lead *C. parapsilosis* to the development of a stronger biofilm in terms of total biomass (Fig. 1a), especially the oral isolate that formed biofilms with higher amounts of viable cells (Fig. 1b) and a direct increase of carbohydrates, specifically β-1,3-glucan was present in the biofilms matrices (Table 2). Likewise, in *C. albicans*, *BCR1* was identified as an important gene required for biofilm formation (Ding & Butler, 2007; Nobile *et al.*, 2006a; Nobile & Mitchell, 2005; Nobile *et al.*, 2006b). Recently, Pannanusorn and co-workers (2013) demonstrated that the transcription factor Bcr1p has an important role in biofilm development in strains with low biofilm formation abilities and a non-essential role in strains with a high level of biofilm formation (Pannanusorn *et al.*, 2013). To provide insight into the role of glucose in *C. parapsilosis* biofilm development the *BCR1* levels of expression were assessed (Fig. 3a). The molecular studies corroborated the in vitro biofilm formation studies (SEM images, Fig. 2), once the increased level of expression of *BCR1* was in accordance with the increased abilities to form biofilms. In addition, high expression levels of *BCR1* were observed in the weaker biofilm producer (*C. parapsilosis* ATCC 20019) comparatively with the low levels of expression observed in the oral isolate *C. parapsilosis* AD, with high capacity to form biofilm.

It is important to highlight that, besides promoting cell proliferation, the high percentage of glucose did not induce yeast toxicity. Nguyen and Nosanchuk, 2011, studied the behaviour of *C. parapsilosis* strains in stress conditions, as high levels of glucose and concluded that yeast cells were able to convert the excessive levels of glucose into lipid droplets. A specific example is the metabolism of fatty acid desaturase that involved Ole1p, which is essential in unsaturated fatty acids (UFA) synthesis (Nguyen & Nosanchuk, 2011). So, in this way the *OLE1* assume an important role in responding to the stress conditions imposed by high glucose concentrations in *C. parapsilosis* (Nguyen et al., 2011b). We demonstrated that the expression of *OLE1* gene was up-regulated with glucose increment (Fig. 3c), which could be explained by an attempt of yeast cells to protect themselves from the toxic effects of free fatty acids generated during *de novo* fatty acid biosynthesis from glucose. On the other hand, some authors have been advocating the importance of this gene in the development of filamentous forms by *C. parapsilosis*, due its direct implications in the fluidity and integrity of yeast membrane

(Nguyen *et al.*, 2011a; b). Interesting, *C. parapsilosis* AD biofilms formed in high glucose media exhibited pseudohyphae (Fig. 2) and the level of OLE1 expression was significantly higher comparatively to that of biofilm cells grown in low glucose contents. This could indicate an adaptation of the oral isolate to the oral environment with hyper alimentation with high levels of sugar. One of the relevant characteristics of biofilms is the presence of an extracellular polymeric matrix, which is generally composed by extracellular DNA, proteins and polysaccharides (Al-Fattani & Douglas, 2006). In this study, biofilm matrix was analyzed concerning total carbohydrate and β-1,3 glucan content (Table 2). Significantly, consistent differences were found in the composition of *C. parapsilosis* biofilms at different concentrations of glucose, with carbohydrates and β-1,3 glucan only detected at the higher concentrations of this sugar (2.0 and 10 %). Silva et al., (2009) reported that matrix of *C. parapsilosis* consisted in high amounts of carbohydrates (Silva *et al.*, 2009a). Moreover, our results demonstrated an increase of carbohydrates and β-1,3 glucan with the increased of glucose in the biofilm formation medium (Table 2). Additionally, *FKS1* gene was used to investigate the role of β-1, 3 glucan matrix delivery. This gene has been shown previously to exhibit and important role on *C. albicans* biofilm formation (Taff *et al.*, 2012). β-1,3 glucan besides being a major component of the fungal cell wall, is also present in the biofilm matrix. The extracellular β -1,3 glucan, has been linked to overall matrix production and drug resistance is catalysed by β-1,3 glucan synthase (GS1) and the gene *FKS1* is responsible for the catalytic subunit of β-1,3 glucan synthase (Nett *et al.*, 2007; Nett *et al.*, 2010; Taff *et al.*, 2012), which substrate is glucose. Our results pointed out that the presence of high levels of glucose up-regulates *FKS1* leading to an increase in β-1,3 glucan synthesis that accumulates in the biofilm matrix contributing to denser and more structured biofilms (Fig. 3).

In summary, despite of the reduced number of the strains, this work demonstrates that glucose is able to modulate the *C. parapsilosis* biofilm, specifically in terms of total biomass, structure and carbohydrates matrix composition. Additionally, it was showed that biofilm related genes like as *BCR1*, *FKS1* and *OLE1* are involved in biofilm modulation by glucose. Nevertheless, the exact mechanism by which glucose enhance carbohydrate production by *C. parapsilosis* biofilms requires future investigation.

ACKNOWLEDGMENTS

This work was supported by the Programa Operacional, Fatores de competitividade – COMPETE and by national funds through FCT – Fundação para a Ciência e a Tecnologia on the scope of the projects FCT PTDC/EBB-EBI/120495/2010, RECI/EBB-EBI/0179/2012 and PEst-OE/EQB/LA0023/2013. The authors thank the Project “BioHealth - Biotechnology and Bioengineering approaches to improve health quality", Ref. NORTE-07-0124-FEDER-000027, co-funded by the Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER.

REFERENCES

Al-Fattani MA & Douglas LJ (2006) Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* 55: 999-1008.

Almirante B, Rodriguez D, Cuenca-Estrella M, Almela M, Sanchez F, Ayats J, Alonso-Tarres C, Rodriguez-Tudela JL & Pahissa A (2006) Epidemiology, risk factors, and prognosis of *Candida parapsilosis* bloodstream infections: case-control population-based surveillance study of patients in Barcelona, Spain, from 2002 to 2003. *J Med Microbiol* 44: 1681-1685.

Akpan A & Morgan R (2002) Oral candidiasis. *Postgrad Med J* 78: 455-459.

Azeredo J, Henriques M, Sillankorva S & Oliveira R (2003) Extraction of exopolymers from biofilms: the protective effect of glutaraldehyde. *Water Sci Technol* 47: 175-179.

Ding C & Butler G (2007) Development of a gene knockout system in *Candida parapsilosis* reveals a conserved role for *BCR1* in biofilm formation. *Eukaryot Cell* 6: 1310-1319.

DuBois M, Gilles KA, Hamilton JK, Rebers PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* 28: 350-356.

Martins M, Henriques M, Ribeiro AP, Fernandes R, Goncalves V, Seabra A, Azeredo J & Oliveira R (2010) Oral *Candida* carriage of patients attending a dental clinic in Braga, Portugal. *Rev Iberoam Micol* 27: 119-124.

Moris DV, Melhem MS, Martins MA, Souza LR, Kacew S, Szeszs MW, Carvalho LR, Pimenta-Rodrigues MV, Berghs HA & Mendes RP (2012) Prevalence and antifungal susceptibility of *Candida parapsilosis* complex isolates collected from oral cavities of HIV-infected individuals. *J Med Microbiol* 61: 1758-1765.

Nett J, Lincoln L, Marchillo K, Massey R, Holoyda K, Hoff B, VanHandel M & Andes D (2007) Putative role of beta-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob Agents Chemother* 51: 510-520.

Nett JE, Sanchez H, Cain MT & Andes DR (2010) Genetic basis of *Candida* biofilm resistance due to drug-sequestering matrix glucan. *J Infect Dis* 202: 171-175.

Nguyen LN, Gacser A & Nosanchuk JD (2011a) Secreted lipases supply fatty acids for yeast growth in the absence of de novo fatty acid synthesis. *Virulence* 2: 538-541.

Nguyen LN, Gacser A & Nosanchuk JD (2011b) The stearoyl-coenzyme A desaturase 1 is essential for virulence and membrane stress in *Candida parapsilosis* through unsaturated fatty acid production. *Infect Immun* 79: 136-145.

Nguyen LN & Nosanchuk JD (2011) Lipid droplet formation protects against gluco/lipotoxicity in *Candida parapsilosis*: an essential role of fatty acid desaturase Ole1. *Cell cycle* 10: 3159-3167.

Nobile CJ, Andes DR, Nett JE, Smith FJ, Yue F, Phan QT, Edwards JE, Filler SG & Mitchell AP (2006a) Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. *PLoS Pathog* 2: e63.

Nobile CJ & Mitchell AP (2005) Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. *Curr Biol* 15: 1150-1155.

Nobile CJ, Nett JE, Andes DR & Mitchell AP (2006b) Function of *Candida albicans* adhesin *Hwp1* in biofilm formation. *Eukaryot Cell* 5: 1604-1610.

Pannanusorn S, Ramirez-Zavala B, Lunsdorf H, Agerberth B, Morschhauser J & Romling U (2013) Characterization of biofilm formation and the role of BCR1 in clinical isolates of Candida parapsilosis. *Eukaryot Cell* 13, 438-451.

Pfaffl MW (2004) Quantification Strategies in real-time PCR. *The Real-Time PCR Encyclopedia A–Z of Quantitative PCR* (Bustin SA), pp. 87-120. La Jolla: International University Line.

Ramage G, Martinez JP & Lopez-Ribot JL (2006) *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res* 6: 979-986.

Santana IL, Goncalves LM, de Vasconcellos AA, da Silva WJ, Cury JA & Del Bel Cury AA (2013) Dietary carbohydrates modulate *Candida albicans* biofilm development on the denture surface. *PloS one* 8: e64645.

Shin JH, Kee JS, Shin MJ, Kim SH, Shin DH, Lee SK, Suh SP, Ryang DW (2002) Biofilm Production by Isolates of Candida Species Recovered from Nonneutropenic Patients: Comparison of Bloodstream Isolates with Isolates from Other Sources. *J Clin Microbiol.* 40 1244–1248.

Silva S, Henriques M, Martins A, Oliveira R, Williams D & Azeredo J (2009a) Biofilms of non-*Candida albicans Candida* species: quantification, structure and matrix composition. *Medical mycology: official publication of the International Society for Human and Animal Mycology* 47: 681-689.

Silva S, Henriques M, Oliveira R, Azeredo J, Malic S, Hooper SJ & Williams DW (2009b) Characterization of *Candida parapsilosis* infection of an in vitro reconstituted human oral epithelium. Eur J Oral Sci 117: 669-675.

Silva S, Henriques M, Oliveira R, Williams D & Azeredo J (2010) In vitro biofilm activity of non-Candida albicans Candida species. Curr Microbiol 61: 534-540.

Taff HT, Mitchell KF, Edward JA & Andes DR (2013) Mechanisms of Candida biofilm drug resistance. Future Microbiol 8: 1325-1337.

Taff HT, Nett JE, Zarnowski R, Ross KM, Sanchez H, Cain MT, Hamaker J, Mitchell AP & Andes DR (2012) A Candida biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog* 8: e1002848.

Trofa D, Gacser A & Nosanchuk JD (2008) *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* 21:606-625.

Zambranoa MLJ, Escribanoa P, Bouza E & Guinea J (2014) Production of biofilm by *Candida* and non-*Candida* spp. isolates causing fungemia: Comparison of biomass production and metabolic activity and development of cut-off points. *Inter J Med Micro* 8:1192-1198.

Williams DW, Kuriyama T, Silva S, Malic S & Lewis MA (2011) *Candida* biofilms and oral candidosis: treatment and prevention. *Periodontol 2000* 55: 250-265.

Williams DW, Wilson MJ, Lewis MA & Potts AJ (1995) Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J Clin Microbiol* 33: 2476-2479.

LEGENDS TO FIGURES

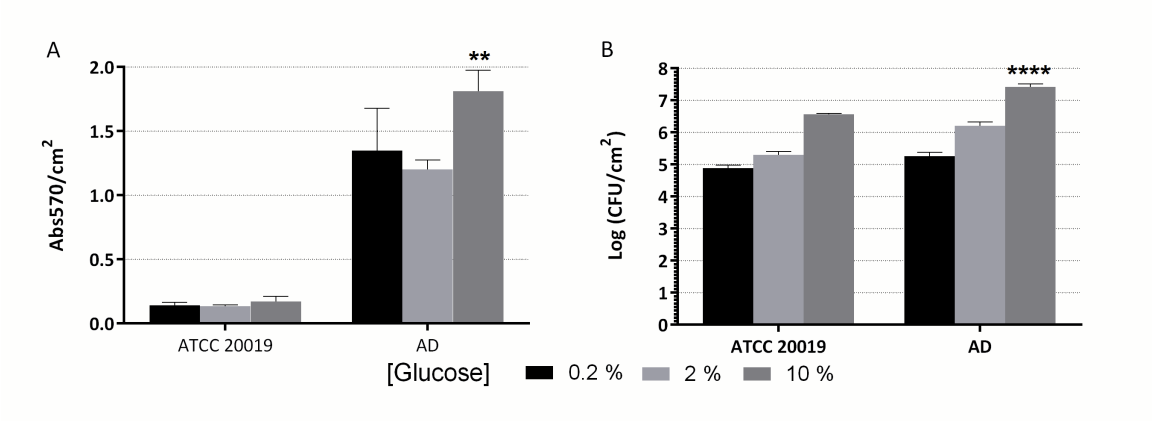

Fig. 1. (a) Absorbance values of crystal violet solutions (A_{570} CV cm^{-2}) and (b) cell viability [Log (CFU cm^{-2})] obtained from 24 h biofilm of *C. parapsilosis* strains formed in RPMI with different concentrations of glucose expressed in % w/v. Error bars represent standard deviation. Statically differences obtained when compared with 0.2 % of glucose (**p<0.01, ****p<0.0001).

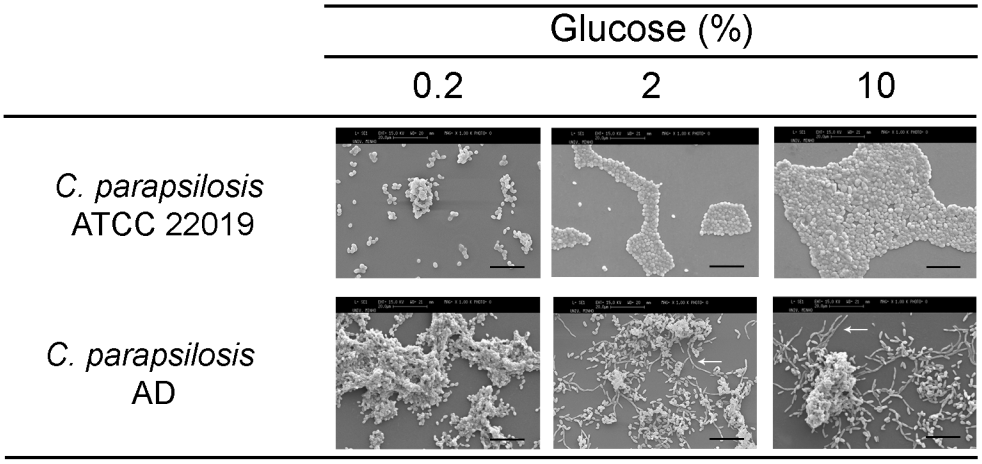

Fig. 2. Scanning electron microscopy images of the *C. parapsilosis* 24 h biofilms formed in RPMI with different concentrations of glucose. Magnification corresponds at 3000 × and the scale bars corresponds to 20 µm. White arrows indicated the presence of pseudohyphal forms.

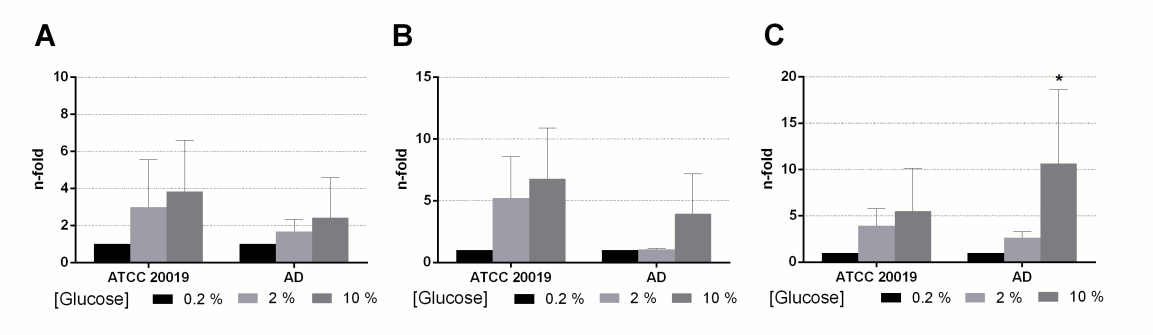

Fig. 3. Level of gene expression (n-fold) of (a) *BCR1*, (b) *FKS1* and (c) *OLE1* in 24 h biofilm of *C. parapsilosis* cells formed in RPMI with different concentrations of glucose. The values were calculated using Pfaffl method, normalized by the expression of the housekeeping gene and compared with the genes expression at the lowest concentration of glucose (0.2 % glucose). Error bars represent standard deviation. Statistically differences obtained when compared with 0.2 % of glucose (*$p<0.1$).

TABLES

Table 1. Primers used for real time-PCR analysis

Species	Sequence (5`- 3`)	Primer	Target
C. parapsilosis	ATCTGCGTTACCCGCAATAC	Forward	*ACT1*
	ATGTCGTCCACAAACAACGA	Reverse	
	CCATTAACCGGGTTGCTATT	Forward	*BCR1*
	GAGTCCGTTATCGCCAATGT	Reverse	
	CGACATCCACATGTCCAATC	Forward	*FKS1*
	CATTGCTGTTGCAACTTTGG	Reverse	
	TGGGGGGTAAACTAATGGGT	Forward	*OLE1*
	TGAAGCACCCATACCAATTG	Reverse	

Table 2. Total carbohydrates and β-1, 3 glucan contents of *Candida parapsilosis* biofilm matrices growth in RPMI with different concentrations of glucose. The values are means ± standard deviations

		Matrix	**Carbohydrate**	**β-1,3 glucan**
Strains	Glucose (% w/v)	$mg_{matrix}\ g_{biofilm}^{-1}$	$mg_{carbohydrates}\ g_{biofilm}^{-1}$	$ng_{\beta\ 1,3\text{-}glucan} mg_{carbohydrates}^{-1}$
C. parapsilosis ATCC 20019	0.2	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
	2	165.6 ± 11.2	13.0 ± 9.6	302.2 ± 130.4
	10	149.3 ± 1.0	22.1 ± 9.7	339.8 ± 9.7
C. parapsilosis AD	0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	2	130.8 ± 81.0	49.6 ± 2.7	261.7 ± 26.9
	10	142.7 ± 110.6	45.7 ± 1.9	333.6 ± 27.7

*Below the threshold detection level