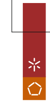

Effect of glucose and glutamine on biofilm formation by several *Candida* species

Bruno Filipe Gomes Ribeiro

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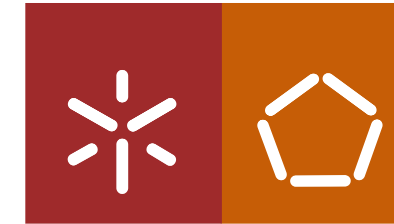

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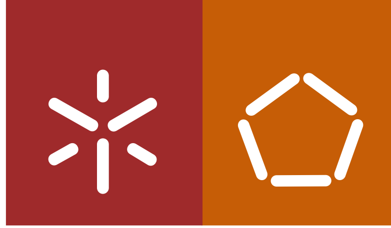

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Effect of glucose and glutamine on biofilm formation by several *Candida* species

Dissertação de Mestrado
Mestrado Integrado em Engenharia Biomédica
Ramo Engenharia Clinica

Trabalho efetuado sob a orientação da
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e da
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outubro de 2013

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Título dissertação

Effect of glucose and glutamine on biofilm formation by several *Candida* species

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Ano de conclusão: 2013

Designação do Mestrado:

Mestrado Integrado em Engenharia Biomédica

Ramo de Engenharia Clínica

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

Universidade do Minho, ____/____/________

Assinatura: ____

Aknowledgments

Gostaria de exprimir a minha mais suprema gratidão à Professora Joana Azeredo, pela disponibilidade, paciência e conselhos dados ao longo deste trabalho, orientando-em sempre na direcção certa.

À minha co-orientadora, Drª Sónia Silva, muito obrigado, pela simpatia, boa disposição, apoio e pelos conselhos e ensinamentos, que me fizeram aprender metodologias que levaram à realização desta tese. Nem nas situações mais difíceis me deixou desanimar.

Ao Carlos Tiago Alves, Leonel Pereira e muitos outros do Departamento de Engenharia Biológica, muito obrigado pelo apoio e pelas orientações.

A todos os colegas que passaram do grupos das Candidas (e também dos fagos), foi um prazer trabalhar no mesmo espaço que vós.

E por fim à minha família, o meu suporte, sempre lá para mim….

Abstract/Sumário

Abstract

Biofilm formation is an important virulence factor that contributes greatly to *Candida* species pathogenicity. This virulence factor is dependent on several environmental factors including the available nutrients. *Candida* species survive in the oral environment through the catabolism of sugars, as glucose, and nitrogen assimilation, through the absorption of certain aminoacids as L-glutamine. There are several genes related with biofilm formation. The *FKS1* gene encodes an membrane bound enzyme that produces β-1,3 glucan, a component of the biofilm matrix, from glucose. *BCR1* gene is a fungal transcription factor that regulates the expression of important *Candida* adhesins. Thus, the aim of this study was to understand the effect of the glucose concentration variation as well as the presence of a nitrogen source (glutamine), in the biofilm formation by several *Candida* species.

Total biomass quantification by crystal violet showed that only the oral isolates of *Candida albicans* and *Candida parapsilosis* biofilm production dependent upon the glucose level in the medium. The oral isolate of *Candida tropicalis* produced more biofilm for smaller amounts of glucose, whereas, *C. glabrata* strains were less able to form biofilm than any other studied strain. Analyzing the effect of glutamine on biofilms in terms of total biomass, only the oral isolate of *C. parapsilosis* was affected by the absence of glutamine from the medium. Scanning-Ellectron microscopy of the biofilms formed by *C. albicans* and *C. parapsilosis*, showed that the complexity and overall structural size of the biofilm increased when the glucose concentration was higher. It also showed, that glucose presence enhanced the development and growth of hyphal/filamentous forms. Genetic expression assessment through Real time PCR, showed that *FKS1* gene expression was glucose dependent for both *C. albicans* and *C. parapsilosis*, and overall higher for *C. albicans*, hinting at a greater β-1,3 glucan incorporation on biofilm of this specie. *BCR1* gene expression increased for higher glucose levels in the medium. However, when glucose was replaced by an equal amount of glutamine, the expression of this gene didn’t decrease for any of the studied strains, hinting that cell adhesion is not dependent on the presence of glucose, but may be dependent on nutrient concentration.

Sumário

A formação de biofilmes é um importante factor de virulência que contribui para a patogenicidade de espécies do fungo *Candida*. Este factor de virulência está dependente de vários factores ambientais incluindo os nutrientes disponíveis. As espécies de *Candida* sobreviveram no ambiente oral através do catabolismo de açucares, como a glucose e assimilação de nitrogénio, através da absorção de certos aminoácidos como L-glutamina. Vários genes estão relacionados com a formação de biofilmes. O gene *FKS1* codifica uma enzima da membrana celular que produz β-1,3 glucano, um componente da matriz dos biofilmes. O gene *BCR1* está relacionado com a capacidade de adesão a superfícies das células. Assim sendo, o objectivo deste estudo foi entender a forma pela qual a variação da concentração de glucose bem como a presença de uma outra fonte de nitrogénio (glutamina), pode afectar a capacidade de formação de biofilmes por várias espécies de *Candida*.

A quantificação da biomassa total mostrou que a produção de biofilme de só os isolados orais de *Candida albicans* e *Candida parapsilosis* foi dependente da quantidade de glucose no meio. O isolado oral de *Candida tropicalis* produziu mais biofilme para concentrações de glucose mais baixas. As estirpes de *Candida glabrata* foram as piores produtoras de biofilme. Analizando o efeito da glutamina na produção de biofilmes, foi possível verificar que só o isolado oral de *C. parapsilosis* foi afectado pela sua ausência do meio. A visualização dos biofilmes formados por microscopia electrónica de varrimento mostrou que tanto a complexidade como o tamanho das estruturas, a nível geral, aumentou com o aumento da concentração de glucose no meio, para estirpes de *C.albicans* e *C. parapsilosis*. Também mostrou que o aumento da concentração de glucose no meio fomentou o desenvolvimento de hifas /estruturas filamentosas para as estirpes destas espécies. A avaliação da expressão do gene *FKS1* através de Real-time PCR mostrou que a expressão deste gene está dependente da glucosa em *C. albicans* e *C. parapsilosis*, sendo, em geral, superior para as estirpes de *C. albicans*. Isto aponta para a existência uma maior quantidade de β-1,3 glucano nas matrizes dos seus biofilmes. A expressão do gene *BCR1* aumentou com a concentração de glucose, mas no entanto não parece ser afectada com a substituição da glucose por uma quantidade equivalente de glutamina no meio.

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Nomenclature

Symbols

pH –hydrogen ion concentration

˚C – Celsium degrees

µm - Micrometer

ml – Milliliters

% - Percent

ml/min – milliliter per minute

cell/ml - cells per milliliter

rpm - rotations per minute

h – hour

min – minute

sec - seconds

µl – microliter

mn – nanometer

abs/cm^2 – absorvance per square centimeter

Log CFU/ cm^2 – Logarithm of the number of Colony formation units per square centimeter

M – molar

mM – milimolar

g – gravity

p – significance value

Abbreviations

Abs – Absorvance

***ACT1* –** Actin 1

AIDS - acquired immunodeficiency syndrome

ALS – Agglutinin-like sequence gene

AMP - adenosine monophosphate

ANOVA – Analysis of varince

ATCC – American type culture collection

ATP - adenosine triphosphate

***BCR1* –** Biofilm cell wall regulator 1 gene

$CaCl_2$ – Calcium chloride

cDNA – complementary Desoxyribonucleic acid

CO_2 – carbon dioxide

CTP - cytidine triphosphate

CV – Crystal violet

DNA – Desoxyribonucleic acid

DNase - desoxirribonuclease

dNTP – Desoxynucleoside triphosphate

ECM – Extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

EPA – Epithelial adhesin gene

GMP - Guanosine monophosphate

GPI - Glycosyl-phosphatidylinositol

HIV - Human immunodeficiency virus

H_2O – water

HWP1 – Hyphal Wall protein 1 gene

IgG - Immunoglobulin G

IgM – Immunoglobulin M

KH_2PO_4 - potassium dihydrogen phosphate

mRNA – messenger Ribonucleic acid

NCAC species – *Non – Candida albicans Candida* species

NAD - Nicotinamide adenine dinucleotide

NRT - negative real time control

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

RNA – Ribonucleic acid

RNAase - Ribonuclease

RPMI – Roswell Park Memorial Institute culture medium

SDA - Sabouraud dextrose agar

SDS – Sodium dodecyl sulfate

SIgA – Salivary Immunoglobulin A

Tris-HCl -Tris-Hidrochloride

TAE - Tris-acetate-EDTA

w/V – weight per volume

1 - Introduction

This chapter consists on a general introduction in which is given a detailed context to the aim of the work developed under this thesis. The latest information concerning *Candida* species biology, epidemiology and biofilm formation is presented.

1.1 - Oral candidosis

Oral candidosis or oropharyngeal candidosis is an opportunistic mycosis of the oral mucosa that, in more extreme cases, can evolve from a superficial infection to a systemic and life threatening infection known as candidaemia (Murray, 1995). The symptoms of candidosis often include local discomfort, an altered taste sensation, dysphagia from esophageal overgrowth. Therefore, it results in a poor nutrition, slow recovery and prolonged hospital stay. In extreme cases, the infection can spread through the bloodstream or upper gastrointestinal tract leading to severe infection with a significant morbidity and mortality rate. Moreover, immunocompromised patients, such as HIV or cancer patients, and diabetes mellitus patients are also specially proponent to candidosis in general (Akpan *et al*., 2002; Epstein, 1990; Guida, 1988; Hoeql *et al*., 1998). There is a diverse number of oral candidosis types. The most common, and most easily diagnosed, is the pseudomembranous candidosis (Figure 1.1 A) or thrush, which accounts for at least a third of the cases. It is characterized by extensive white pseudomembrane consisting of desquamated epithelial cells, fibrin, and fungal hyphae (Akpan *et al*., 2002).

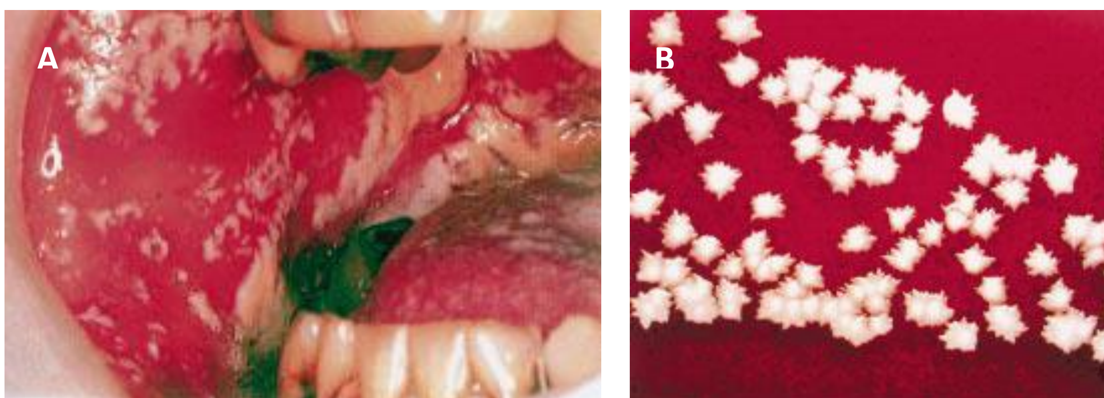

Figure 1.1 – Different aspects of Oral candidosis. A) Aspect of acute Pseudomembranous Candidosis or trush, the most easily identifiable type of oral candidosis and tradicionally associated with this disease; B) Candida albicans colonies aspect seen under light microscopy (adapted from Akpan *et al*. 2002).

Accutic atrophic candidosis is associated with a burning sensation in the mouth or on the tongue and is of difficult diagnosis, which is helped by a swab of the tongue. *Chronic hyperplastic candidiasis*, is characterized by several white lesions that occur in the buccal mucosa or lateral border of the tongue. However, because *Candida* species, are not frequently isolated from clinical sites of the disease, it is believed that this condition may be confused with others. *Chronic atrophic candidiasis* also known as denture stomatitis is characterized by localized chronic erythema of tissues covered by dentures. Lesions can also occur on the palate, upper jaw and mandibular tissue. *Median rhomboid glossitis* is a chronic symmetrical area on the tongue anterior to the circumvallate papillae. It is made up of atrophic filiform papillae. *Angular cheilitis* is an erythematous fissuring at one or both corners of the mouth and is usually associated with an oral *candida* infection.(Akpan *et al.*, 2002)

1.1.1 – Causative agents of oral candidosis

Oral candidosis is caused by the overgrowth or infection of the oral mucosa by a *Candida* fungus. Several species of *Candida*, like *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata*, among others, have been identified in the isolates of clinical infection. These, however, are also commensal microorganisms that can be found in the normal flora of humans, as the oral, gastrointestinal or rectal cavities, and generally causes no problems to healthy people (Akpan *et al*., 2002; Hoeql *et al.*, 1998; Murray, 1995). They can also be found in environmental surfaces, and infections can also originate from exogenous sources (Lass-Flörl, 2009). From sampling and isolation, it has been determined that, in healthy adults and children, there was a median carriage frequency of 34.4% in general of yeasts and from those, 17% were from *C. albicans* alone. *C. tropicalis*, *C. glabrata* and *C. parapsilosis* followed in dominance (Odds, 1988). Almost 50% of healthy and disease humans carrie commensally *C. albicans* (Shimizu *et al.*, 2007).

1.1.2 – Predisposing factors

Clinical observations suggested that nutritional factors can affect, either locally or systemically, the pathogenicity of oral candidosis (Akpan *et al*., 2002; Samaranayake, 2006). According to various studies, the presence of certain dietary carbohydrates in the growth medium, can influence the superficial adhesion of the yeasts, the development of biofilms, morphogenesis, enzyme production and activity and growth over time (Samaranayake *et al.*, 1982; Santana *et al.*, 2013). Population based surveillance of candidosis conducted in USA, Canada and Europe revealed that the highest rates of the desease were registered to infants with less than 1 year of age and in adults over the age of 65 have been reported in the US studies. It is also documented a high incidence of candidosis among cancer patients and adults with diabetes as well as the near universality of central venous catheters among patients diagnosed with candidaemia (Pfaller *et al.*, 2007). The widespread use of broadspectrum antibiotics and invasive medical devices, the rising number of immunocompromised and HIV patients, and the increase of the elder population have contributed to the increase in the incidence of candidosis infections (Fanello *et al.*, 2001; Kojic *et al.*, 2004; Silveira *et al.*, 2007). The breach of the mucosal barrier or the insertion of contaminated syringes can cause infection, by giving hematogenous access to fungus. An insufficient salivary flow, for example, due to drug action, radiotherapy or Sjgnören's syndrome, diminishes the dilutional effect of saliva, and so, the removal of these microorganisms from the mucosa as well as the effect of antimicrobial proteins like lactoferrin, sialoperoxidase, lysozyme, hisitidine-rich polypeptides, and specific anticandida antibodies that can be found in saliva (Akpan *et al*., 2002). Drugs, such as inhaled steroids, are suspected of suppressing cellular immunity and phagocytosis of the local mucosa (Akpan *et al*., 2002; Ellepola *et al.*, 2001). The use of dentures provides a microenvironment, in the denture fitting, with low oxygen, low pH and an anaerobic environment, in which there is a reduced salivary flow. This, aided by the great affinity of *Candida* species to acrylic and poor oral hygiene leads to overgrowth and infection (Akpan *et al*., 2002; Henriques *et al.*, 2004).

Table 1.1 – Predisposing factors to oral candidosis

Factor	Description	References
Age	Under 1 year and above 65 years;	(Akpan *et al*., 2002) (Douglas, 2003)
Immunodeficiency	HIV patients; Cancer patients including the ones under chemotherapy; Drugs; Reduced salivary flow;	(Akpan *et al*., 2002) (Douglas, 2003) (Odds, 1988)
Invasive treatments	Syringes; Catheters; Trauma cirurgy patients;	(Akpan *et al*., 2002) (Wisplinghoff *et al.*, 2004) (Negri *et al.*, 2011)
Oral sugars	Reduced salivary flow; Radiotherapy; Signören's syndrome; Xerostemia; Diabetes;	(Akpan *et al*., 2002) (Hill *et al.*, 1989) (Silva *et al.*, 2012) (Andrés *et al.*, 2008)
Dentures	High adhesion to denture surfaces; Environment with low oxygen and pH;	(Akpan *et al*., 2002) (Henriques *et al*., 2004) (Douglas, 2003)

1.1.3– Incidence rates

In the last decades, there has been a rise in the incidence of *Candida* infections. The report of Epidemiologic surveillance of bloodstream infections, of 2010 by the health quality department in Portugal, stated that 61.9% of all bloodstream fungal infections were related to *Candida* alone (Pina *et al*., 2010). In the United States, *Candida* infections are the fourth most common cause of all nosocomial bloodstream infections, representing 8 to 10% of all bloodstream acquired in the hospitals (Pfaller *et al*., 2007; Wisplinghoff *et al*., 2004). Population-based surveys, conducted in the US, between 1996 and 2003, in several major cities as San Francisco, Atlanta showed an

incidence of 8 per 100000 population, while surveys conducted in Iowa, Baltimore and Connecticut reported a incidence of 24 per 100000. Rates of 6-14 per 100000 have been reported in average, across the country. In Europe, among the Nordic countries, Norway, Finland and Sweden reported incidence of candidaemia around 3 per 100000 population whereas Denmark reports 11 per 100000 population. In the middle and southern parts of Europe the UK, Scotland, Spain and Italy reported 1.2-6.4 per 100000 population. The results showed not only that the incidence rates are significantly more superior in the United States than in Europe, but also that generally there was an increase in the incidence rates over the years (Arendrup, 2010; Pfaller *et al*., 2007).

1.1.4 – Emergence of Candida Non-Candida albicans as pathogens

Despite of most of the candidosis infections being attributed to *Candida albicans*, in the last three decades there has been an increase of cases caused by Non-*Candida albicans Candida* (NCAC) species, such as *C. parapsilosis*, *C. tropicalis* and *C. glabrata*. Brachiesi *et al.* reported that the frequency of isolation of NCAC from clinical sites, raised from 3-4% in 1988-1989 to 16-18% 1990-1991 (Brachiesi *et al.*, 1993). Masiá *et al* realized that 21% of HIV patients with diagnosed candidiasis, were infected with NCAC (Masiá *et al.*, 1999). More recently, Ventura *et al.* while studying the species and susceptibility in several candidaemia cases in a large Brazilian hospital, realized that 51.6% of the cases was caused by a *NCAC* pathogen (Ventura *et al.*, 2004). Lass-Flörl, J in a review about the changing epidemiology of fungal infections in Europe, reported that despite of *Candida albicans* still be responsible for more than half the cases of candidaemia in Europe, the number of *NCAC* species-related candidaemia cases are rising. 14% of the cases were caused by *C. glabrata* or *C. parapsilosis* each and 7% of them by *C. tropicalis*.(Lass-Flörl, 2009). Globally, *C. albicans* is still the major pathogen, being related to 50-70% of the cases of invasive candidaemia worldwide, although these values are lower than a few decades ago (Arendrup, 2010).

The causes for this change in *Candida* epidemiology may be related to an increase in the incidence of *Candida* oral infections due to the advent of immunodeficiency virus infection (AIDS), as well as immunosuppressive treatments for certain diseases, as cancer, which debilitated the host immune control of commensal *Candida* populations, leading to overgrowth. The advent of new medical procedures, the rise in

the use of invasive medical procedures and devices and the widespread use of broad-spectrum antibiotics also contributed to an increase in *Candida* incidence.(Odds, 1988) On the other hand, the development of new diagnostic technics that implemented molecular and serological techniques into the identification of *Candida*, lead no only to a more accurate diagnosis of the disease, which was mistaken for other similar conditions, as well as a more accurate identification of *Candida* specie and strain.(Calderone, 2002; Suzuki, 2002)

1.2 - *Candida* species: growth and metabolism

There are about 350 different species in the genus *Candida*, which belongs to the Fungi kingdom. From those, however, only a small percentage is pathogenic to humans, being 65% unable to grow at a temperature of 37˚C. Over the years, many taxonomic changes led to not only the rename of some *Candida* species into new ones, but also the addition of more elements to the family, recently discovered or reclassified into new taxonomic branches. Initially, *Candida* species without a sexual stage were classified as Deuteromycota. The species with a sexual stage, on the other hand, belonged to the phylum of the Ascomycota. Later genetic studies proved a phylogenetic relationship between the organisms that presented or not a sexual stage, thus validating the alignment of *Candida* spp. among the Ascomycota. Besides the genetic approach, there were other characteristics that distinguished *Candida* species as Ascomycota, as the fact that they are urease negative, non-encapsulated, fermentative, and non-inositol assimilative and the production of β-glucans into their cell wall (Calderone, 2002; Kourkoumpetis *et al.*, 2010; Odds, 1988).

1.2.1 – Growth forms of *Candida* species

Candida species generally grow as yeasts and most of them can also produce hyphae or pseudohyphae (filamentous type of growth). The main difference between hyphae and pseudohyphae is related to the way in which they are formed. Pseudohyphae are formed form yeast cells or from hyphae by budding, but the new growth remains attached to the parent cell and is elongate, resulting in filaments with constrictions at the cell-cell junctions. There is no internal cross walls (septa) associated with pseudohyphae. Pseudohyphae are chains of more or less elongated yeast cells joined end to end. Budding occurs laterally, just behind the septa, the latter of which are perpendicular to the main axis. True hyphae, however, forms from existing cells or branches and grows by apical extension, firstly as a germ tube, and later, cross walls are formed behind the growing tip of the hyphae (Calderone, 2002; Gow, 2002).

Candida albicans is able to grow in yeast form but also to produce pseudohyphae and/or true hyphae, being truly polymorphic (Calderone, 2002; Gow, 2002). *Candida*

parapsilosis cannot produce true hypha, being able to grow only as yeast or as pseudohypha (Laffey *et al.*, 2005). However, *Candida parapsilosis* pseudohypha are large and curved when compared to other *Candida* species. *Candida glabrata* is reportedly able to grow only as yeast. These species used to be classified in the genus of *Torupsis* precisely due to the lack of a pseudohyphal phenotype, before being assigned to the genus *Candida* (Fidel *et al.*, 1999). Csank *et al.*, however proved that *Candida glabrata* was able produce pseudohypha when grown in a solid nitrogen starvation media (Csank *et al.*, 2000). *Candida tropicalis* grows in yeast form, producing oval blastospores, and also in pseudohyphal form. It is also capable of producing true hypha (Calderone, 2002; Moralez *et al.*, 2013; Okawa *et al.*, 2006).

Several studies have been made in order to identify the factors that induce morphogenesis in *Candida* species. It is known that at a temperature of 37˚C, at a neutral pH, and in the presence of specific nutrients in the growth medium, are implicated in the dimorphic regulation (Gow, 2002). *Candida albicans*, at a temperature above 35˚C, a pH greater than 6.5 and in situation of nitrogen and/or carbon starvation, can produce true hypha. Besides these factors, other parameters as low oxygen concentration, non-fermentable carbon sources and the presence of a wide range of components as proline and other aminoacids, some human hormones or certain alcohols have been identified as factors that promote true hypha development. Yeast cell growth, by the other hand is promoted with an inoculum superior to 10^6 cell/ml^{-1}, a temperature below 35˚C, a pH inferior to 6.5, glucose and ammonium salts (Brown, 2002). A summary of the morphologic difference between *Candida* species is presented below (Calderone, 2002).

Table 1.2 – Morphologic characteristics of *Candida* species (adapted from Calderone, 2002)

Species	Germ tube	True hyphae/Pseudohyphae	Yeast size (µm)
Candida albicans	+	+/+	4-6 X 6-10
Candida parapsilosis	-	-/+	2.5-4 X 2.5-9
Candida glabrata	-	-/-	1-4
Candida tropicalis	-	±/-	4-8 X 5-11

1.2.2 – Metabolism of *Candida* species

Candida is a fungus and like other fungus, they are non-photosyntetic, eukaryotic organisms, which obtain their energy by metabolizing several chemical compounds. Glucose is the main carbon source of *Candida* spp. although they all are capable of metabolizing others sugar as galactose and fructose (Calderone, 2002; Gow, 2002; Murray, 1995). Therefore polymeric sugars as lactose, sucrose and maltose, can equally be metabolized by *Candida* spp. The breakdown of these organic compounds allows the fungus to obtain energy in the form of ATP. *Candida albicans* is able to assimilate and ferment a wide variety of sugar, except sucrose. *Candida glabrata* ferments and assimilates only glucose and trehalose. *C. tropicalis* has the ability to ferment and assimilate sucrose and maltose. Curiously, *C. parapsilosis* was firstly include as a specie of *Monilia*, due to its inability to ferment maltose (Odds, 1988; Trofa *et al.*, 2008).

Candida metabolism happens mainly in two distinct pathways, in which occurs conversion of glucose to pyruvate, with associated glucose formation. In the presence of oxygen, pyruvate, enters the mitochondrial organelle and is decarboxylated in the citric acid cycle, producing both ATP and CO_2. In the absence of oxygen, pyruvate is decomposed, by pyruvate decarboxylase in ethanol, CO_2 and oxaloacetate. *Candida* cells populations grow in aerobic conditions but cannot grow in anaerobic conditions. However, grown populations of *Candida* species can adapt to anaerobic conditions (Gow, 2002).

Glutamine is an aminoacid that plays an essential role for a variety of cell types of the human organism, and a large number of tissues in the body. It acts as a precursor for neurotransmissors, immune-function and acid-base balance, among others. Is the most abundant extracellular aminoacid in vivo, being principally found, at high concentrations, in the blood plasma and skeleton-muscle. It is used as alternative energy source for several fast growing cells, as tumoral cells. (Aledo, 2004; Newsholme *et al.*, 2003) In Candida species, glutamine can act as nitrogen source, and is required as a prominent precursor in several important pathways as the synthesis of tryptophane, asparagine, histidine, phosphate, CTP, AMP, GMP and NAD (Gow, 2002; Odds, 1988).

1.3– The oral cavity and the oral environment

In the oral cavity, there are several habitats characterized by different physicochemical factors, in which proliferates a wide range of microbial communities. This is partly due to the great anatomical diversity of the oral cavity and the interrelationship between the different anatomic structures. The oral cavity possesses both hard and soft tissues, namely, the tooth and the mucosa and in general, is a moist environment at a relatively constant temperature (34 to 36˚C) and a pH close to neutrality in most areas. The tooth is a hard surface, that offers many sites of colonization, both above and below the gingival margin. The oral mucosa, on the other hand, is a soft surface characterized of undergoing constant desquamation, which allows the rapid elimination of adhering microorganisms. The mucosa covers the cheek, the tongue, the gingiva, the palate and the floor of the mouth. However, it varies according to the anatomical site. For example, in the palate the epithelium is keratinized while in gingival crevice, it is not. The mucosal surfaces are bathed by saliva, with the exception of the gingival crevice which is bathed by another physiological fluid, the gingival crevicular fluid, which is an exudate originated from plasma that baths only tooth and gingival from the junctional epithelium in gingival crevice (Marcotte *et al.*, 1998).

1.3.1 – Ecology of the oral cavity

The oral microbiota of the human mouth is composed by a wide and complex variety of species. There are more than 300 species of bacteria alone, to which must be added protozoa, yeasts, and mycoplasmas. This so high diversity of microorganisms may be due to the great anatomical diversity of the mouth, which contributes to the existence of a diverse number of different physicochemical niches. Specie distribution varies quantitatively according to the habitat (Marcotte *et al*., 1998).

On the teeth, microorganisms form a dense mass called a dental plaque, which consists of microbial communities organized in a complex matrix composed of microbial extracellular products and salivary compounds. This plaque forms preferentially on surfaces protected from friction as the area between two adjacent teeth, the gingival crevice, or the bits and fissures on the upper surfaces of the tooth,

responsible by biting. The majority of the organisms isolated from dental plaque are fermentative anaerobic gram-positive bacteria. Gram-negative bacteria may also be isolated but in lower proportions (Marcotte *et al*., 1998).

The oral mucosa of the gingiva, palate, cheeks and mouth floor is colonized with few organisms. *Streptococci* constitutes the highest portion of them. On the mucosa of the tongue, a higher bacterial density and diversity is found (Marcotte *et al*., 1998). *Candida albicans* primarily colonizes the tongue, flowing into the oral mucosa, tooth surfaces, the biofilm and the saliva. Its pathogenic action is favored by local or systemic changes (Silva *et al*., 2012). Comparatively, *Candida glabrata* has lower keranocyte-adherence capacity, but higher adherence ability to surface of dentures, thus possessing a lower capacity of adhesion and invasion of the oral mucosa (Li *et al.*, 2007). However, it has already been reported a high capacity of co-infection of human oral epithelium of this specie with *C. albicans* (Silva *et al.*, 2011). Both *Candida parapsilosis* and *Candida tropicalis* have a great adherence and invasion capacity to the oral epithelium (Meurman *et al.*, 2007).

1.3.2 – Microbial growth-influencing factors

The growth of microorganisms, including *Candida* species in the oral cavity is influenced by a great number of factors. These include temperature, oxidation-reduction potential, pH and the availability of nutrients (dietary sugar intake), water, the anatomy of oral structures, salivary flow, and antimicrobial substances. Each factor in a given oral habitat influences the selection of oral microorganisms (Meurman *et al*., 2007).

1.3.2.1 – Temperature

The temperature of the oral mucosa is relatively constant (34 to 36˚C), which allows a wide range of microorganisms to grow. The temperature may be more variable on the mucosal and tooth supragingival surface. During food intake, microorganisms colonizing these sites are exposed to hot and cold meals and probably must adapt to these extreme variations of temperature (Meurman *et al*., 2007).

1.3.2.2 - pH

The pH or hydrogen ion concentration of an environment affects microorganisms and microbial enzymes directly and also influences the dissolution of many molecules that indirectly influence microorganisms. Microorganisms generally cannot tolerate extreme pH values. In the oral cavity, the pH is maintained near neutrality (6.7 to 7.3) by saliva. The saliva contributes to maintenance of the pH by two mechanisms. First, the flow of saliva eliminates carbohydrates that could be metabolized by bacteria and removes acids produced by bacteria. Second, acidity from drinks and foods, as well as from bacterial activity, is neutralized by the buffering activity of saliva. Bicarbonate is the major salivary buffering system of saliva, but peptides, proteins, and phosphates are also involved. Increases in pH also result from bacteria that metabolize saline and urea into ammonia. Acids that are produced by the microbial metabolism of carbohydrates may accumulate in dental plaque because of the slow diffusion of saliva through it. Following sugar intake, the pH of dental plaque may decrease to below 5. A high concentration of sugar in the oral environment, due, for example, to frequent sugar intake leads to the growth of acidosis bacteria whose carbon metabolism may result in a decrease of the environment pH (Marcotte *et al*., 1998). Candida species carbohydrate metabolism also result in acid production (Samaranayake, 2006).

1.3.2.3 – Dietary sugar intake

Microorganisms living in the supragingival environment have access to nutrient from endogenous sources or exogenous sources. Several components in saliva, as water, carbohydrates, glucoproteins, proteins, aminoacids and various ions as potassium, calcium, chloride, bicarbonate and phosphate, are important nutrients to *Candida* species development. Moreover, exogenous, dietary carbohydrates and proteins have the greatest influence on the composition of the oral microbiota (Marcotte *et al*., 1998). Exogenous glucose is rarely found in food in its single molecular form, being found in most dietary carbohydrates, either as their only building block, which is the case of starch and glycogen, or associated with other monosaccharides as fructose and galactose, forming respectively sucrose and lactose. Grain product such as bread, past, cereals and rice, fruits and vegetables, milk and

duiary products, meat, table sugar, honey among others are foods that contain glucose (Konig, 2000; Samaranayake, 2006). Glutamine can also be found in the composition of the aforementioned foods, besides existing on saliva, in residual concentrations, due to the contributions of the crevicular fluid from blood.

Glucose concentration in saliva may vary due to several factors that may include food intake, mastication, salivary flow, oral hygiene and diabetes. Salivary glucose concentration after the drinking of a glass of water is around 1.38% a value that increases on diabetics to 3.37%, in gram per liter, to man and woman alike. During physiologic operations in the mouth as mastication, the salivary flow increases and the glucose concentration of saliva in the oral environment may decrease or even increase (Jurysta *et al.*, 2009). In fasting conditions, the glucose level in saliva is estimated to be 1.23% for healthy individuals, and 4.22% in diabetic individuals. Age and gender do not appear to be relevant. However, the blood-serum glucose concentration has a direct relation to the salivary glucose concentration (Abikshyeet *et al.*, 2012). Panchbhai *et al*. estimated a glucose concentration of 6.66% for individuals with controlled diabetes, 8.11% for individuals with uncontrolled diabetes and 1.98% for healthy individuals after saliva swallowing (Panchbhai, 2012). A decreased salivary flow rate in diabetes mellitus individuals, can lead to an higher accumulation of sugars in the oral cavity (Jawed *et al.*, 2011). Cases of xerostemia, or dry mouth, are common among diabetic individual, and are directly associated with low salivary flow rates. The normal unstimulated flow rates are about 0.3 to 0.4 ml/min. In stimulated flow rate, as in mastication, the flow rate increases to 1 to 2 ml/min in normal conditions and in diabetes patients without xerostomia, those values decreased considerably to 0.12 ml/min in unstimulated conditions, being equal to the minimum acceptable to the stimulated flow. In such conditions, healthy people, would complain of dry mouth. In the cases of xerostemia diabetic patients, the salivary flow rates were abnormally low, with values from 0 to 0.05 ml/min (Sreebny *et al.*, 1992).

1.4 - *Candida* species biofilm formation

Pathogenic *Candida* species have an already known number of virulence factors. The ability to adhere to medical devices and/or host cells, biofilm formation, dimorphism and secretion of hydrolytic enzymes, such as proteases, phospholipases and lipases, and haemolysins (Silva *et al.*, 2009; Yanq, 2003). This allows the microorganism to colonize the tissue, to degrade it, and to gain access to the blood stream and nutrients. Biofilm formation, particularly, occurs after initial attachment of the fungus to the host cells and/or medical devices. Cells then divide, proliferate and subsequently, biofilm is formed. Biofilms are described as surface associated communities of microorganisms embedded within an extracellular matrix. It confers significant resistance to antifungal therapy by limiting the penetration of substances through the matrix and protecting cells form host immune responses. High mortality rates have been associated with strains of *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* capable of forming biofilms, when compared with strains incapable of forming biofilms (Silva *et al*., 2011).

1.4.1 – The first steps of biofilm formation

Cell adherence to the host cell, or abiotic medical device surface is the first step in the infection by *Candida* spp. From surface adhesion, the cell proliferates and can potentially form biofilms. It is in the cell wall of the *Candida* spp. that physicochemical interactions with some specific components with host/abiotic surface can occur. Some cell wall glycoproteins or polyssacharides are directly involved in surface adhesion (Calderone, 2002; Silva *et al*., 2011). In *C. albicans* there is a family of nine proteins, encoded by genes of the *ALS* family, that can adhere to endothelial and epithelial cells.(Hoyer, 2001) Hwp1, is a hypha cell wall protein linked by a GPI anchor to the cell wall, that is capable of linking small proline rich proteins, found in the surface of *C. albicans*, to the keratinized surface of the human mucosa (Hoyer, 2001). *C. glabrata* possesses a family of 23 proteins, that is encoded by the *EPA* gene family, and that are capable of mediating adherence to epithelial cells (Kaur *et al.*, 2005).

Hydrophobicity of the yeast cell surface is another determining factor for a successful surface adherence. The initial adhesion of *C. parapsilosis* cells is associated

with surface hydrophobicity. It was demonstrated that these species had a greater ability to adhere to epithelial cells and acrylic than *C. albicans* (Panagoda *et al.*, 2001). Hazen *et al*. demonstrated that there was comparable degree of hydrophobicity between, a limited number of isolates of *C. albicans* and *C. glabrata* when grown in specific conditions (Hazen *et al.*, 1986). Contradictorily, however, Kikutaní *et al*. demonstrated that in some growth conditions there was a high variability of the hydrophobicity of *C. albicans* while the hydrophobicity of several isolates of *C. glabrata* remained relatively constant (Kikutani *et al.*, 1992). Once adhered to the epithelium, *Candida* species have the ability to secrete enzymes, proteases and phospholipases that contribute to tissue invasion by this species. Secreted aspartyl proteinases possessed the capability of degrading important immunological and structural defense proteins, disrupting the host mucosal barrier and invading the human epithelium. Phospholipases are capable of degrade phospholipid into fatty acids, damaging also host-cells membranes and increasing the ability to invade and damage the human tissues (Silva *et al*., 2011).

1.4.2 – Biofilm formation

Biofilm formation is a virulence factor of *Candida albicans*, *Candida parapsilosis*, *Candida glabrata* and *Candida tropicalis* species and is linked to the high mobility and mortality (Negri *et al.*, 2012; Negri *et al*., 2011; Silva *et al*., 2009; Silva *et al.*, 2010; Silva *et al*., 2011). Biofilm formation begins by the adhesion of the cells to a surface, through the mechanisms previously described. The cells proliferate, and not much later, distinct microcolonies are formed. If the species are able to form true hyphae, then the first germ tubes start forming after 3-6h (Chandra *et al.*, 2001; Hawser *et al.*, 1994). After 12-14h, the cell colonies, who have grown and aggregated, began to be covered by the extracellular matrix (ECM) produced by the cells (Chandra *et al*., 2001). After 18-24h, the population increased to high number being composed by yeats, pseudohypha, or hypha. Until 48h the biofilm is considered mature, producing higher quantities of the ECM (Hawser *et al*., 1994). It is assumed that the formation of mature biofilms and subsequent production of extracellular matrix is strongly dependent upon species, strain, and environmental conditions as pH, medium composition and oxygen availability.

Candida albicans biofilm formation is associated with the dimorphic switch from yeast to hyphal growth (Donlan *et al.*, 2002). Invasive *C. albicans* strains form more biofilm than non-invasive strains, but the metabolic activity of the biofilms of non-invasive strains is higher. *Candida albicans* has a greater ability to form biofilm than other *Candida* species, such as *Candida parapsilosis*. *Candida parapsilosis* biofilms in general are smaller and produce less ECM than *C. albicans* biofilms. In contrast to *C. albicans*, *C. parapsilosis* biofilms are thinner, less structured, and consist exclusively of aggregate blastospores (Kuhn *et al.*, 2002). Interestingly, however, biofilms formed by several different clinical-isolated strains of *C. parapsilosis* presented a great heterogeneity in biofilm formation. Oral isolates of *C. parapsilosis* are actually more capable of forming biofilms than several strains of *C. tropicalis* and *C. glabrata* (Silva *et al*., 2009). *Candida parapsilosis* cells are able to form biofilms in a diversity of clinical medical devices when exposed to higher glucose concentration, having a great prevalence in bloodstream infections and parental nutrition patients (Kuhn *et al*., 2002; Trofa *et al*., 2008). Therefore, in growth media containing higher concentrations of glucose and/or lipids, *C. parapsilosis* has an expeditious capacity of forming biofilms. The selective preference of this species for plastic medical devices is of particular interest, as biofilm formation enhances the capacity of the organism to colonize catheters and intravascular cellular lines (Trofa *et al*., 2008). *Candida tropicalis* clinical isolates have been classified as being strong biofilm formers, and the biofilm formation seems to be homogenic between different strains (Negri *et al*., 2011; Silva *et al*., 2009). *Candida glabrata* biofilm formation ability has been reported to be more reduced when compared with other *Candida* species, when grown in rich culture media. Biofilm formation ability also appears to be homogeneous between different strains of this specie (Silva *et al*., 2009).

1.4.3 – *Candida* species biofilm structure and composition

The biofilm matrix is composed, in generally by carbohydrates, glucose, proteins, hexosamine, phosphorus and uronic acid. *Candida albicans* biofilms are composed mainly by carbohydrates and glucose, and small amounts of protein, hexosamine, phosphorus and uronic acid. *C. tropicalis* biofilms consisted mainly in hexosamine, with small amounts of carbohydrates and glucose, protein and

phosphorus (Al-Fattani *et al.*, 2006). Comparatively to other *NCAC* species, *C. tropicalis* has the lower amount of both protein and carbohydrate. However, the quantitative composition of the biofilm for this specie is strain dependent. *Candida parapsilosis* biofilms composition is strain dependent having, in general, high amounts of carbohydrates, and low amounts of protein (Silva *et al*., 2009). *Candida glabrata* biofilms have relatively high amounts of carbohydrates and protein, being the protein levels several times higher than those from *C. albicans* and *C. parapsilosis* (Silva *et al*., 2009).

Candida albicans biofilm structure involves, generally, two distinct layers: a thin, basal yeast layer and a thicker, less compact hyphal layer (Donlan *et al*., 2002). The basal layer is covered by a thick, biphasic matrix consisting of extracellular components comprised of cell-wall like compounds and abundant hyphal elements. *C. parapsilosis* biofilms are shallower, less thick and less compling than *C. albicans* biofilms (Kuhn *et al*., 2002). They consisted only on a non-continuous aggregate of cells. However, the biofilm structure for this species is highly strain dependent, and some oral isolates biofilms, not only are constituted by a thick layer of aggregated yeast and pseudohyphal cells, but also presented a multilayered and compact structure that covers the entire surface(Silva *et al*., 2009). *C. tropicalis* biofilms were also strain dependent with some strains possessing a thick biofilm of co-aggregated cells, sometimes with particularly long hyphal elements, while for other strains biofilms were a discontinuous monolayer of yeasts anchored to the surface. *Candida glabrata* biofilms were either a thick multilayer biofilm structure or clusters of several cells. This one was valid for *C. glabrata* ATCC 2001 strain and *C. glabrata* D1 strain (Silva *et al*., 2009).

1.6 - Genes *BCR1* and *FSK1*

One key component present in *Candida albicans* and *Candida parapsilosis* biofilms is β-1,3 glucan. It is one of the main components of the cell wall of *Candida* species, being synthesized by a membrane-bound enzyme, the β-1,3 glucan synthase from UDP-D-glucose (Chauhan *et al.*, 2002). The UDP-D-glucose forms from glucose combination with phosphate and later with urydil transferase (Lee *et al.*, 2003). β-1,3 glucan synthase is a transmembranar, multisubunit enzyme complex, which is activated by GTP, and has been fractionated into two components, the GTP-bound component and the membrane bound component, also known as catalytic component. The catalytic component is encoded by two isogenes, the *FKS1* and the *FKS2* (Chauhan *et al*., 2002).

Three enzymes are involved in the delivery and incorporation of β-1,3 glucan from the cell wall to the biofilm matrix. BGL2p glucanosyltransferase, the PHR1p glucnaosyltransferase e a XOG1p glucanosyltransferase. It is supposed that these enzymes release and modify cell wall glucan for deposition in the extracellular space. An alternative explanation is that the enzymes act in the extracellular space, contributing to steric changes in glucan that are important for mature matrix organization and function. Deletions in the *PHR1* gene reduce cell adhesion and biofilm formation. Deletion in the genes *PHR1*, *XOG1* and *BLG2* reduce up to 10-fold the glucan concentration in the matrix. It also, augments biofilm susceptibility to antifungal agents, and fluorescence test proves that in the gene deletion cases the biofilm capability to sequester the drugs, which supports the idea that this proteins have a key role in glucan incorporation (Taff *et al.*, 2012).

The pathway is ilustrated in figure 2. Basically, the three enzymes, Bgl2, Xog1 and Phr1 have a complementary role in glucan modification. The process of glucan modification involves glucan hydrolysis and the formation of new branch linkages. This enzymes act independently of previously identified Zap1, a transcription factor known to function in matrix production. The overall expression of the *ZAP1* gene have no kind of relation with *XOG1*, *PHR1* or *BGL2* gene expression (Taff *et al*., 2012).

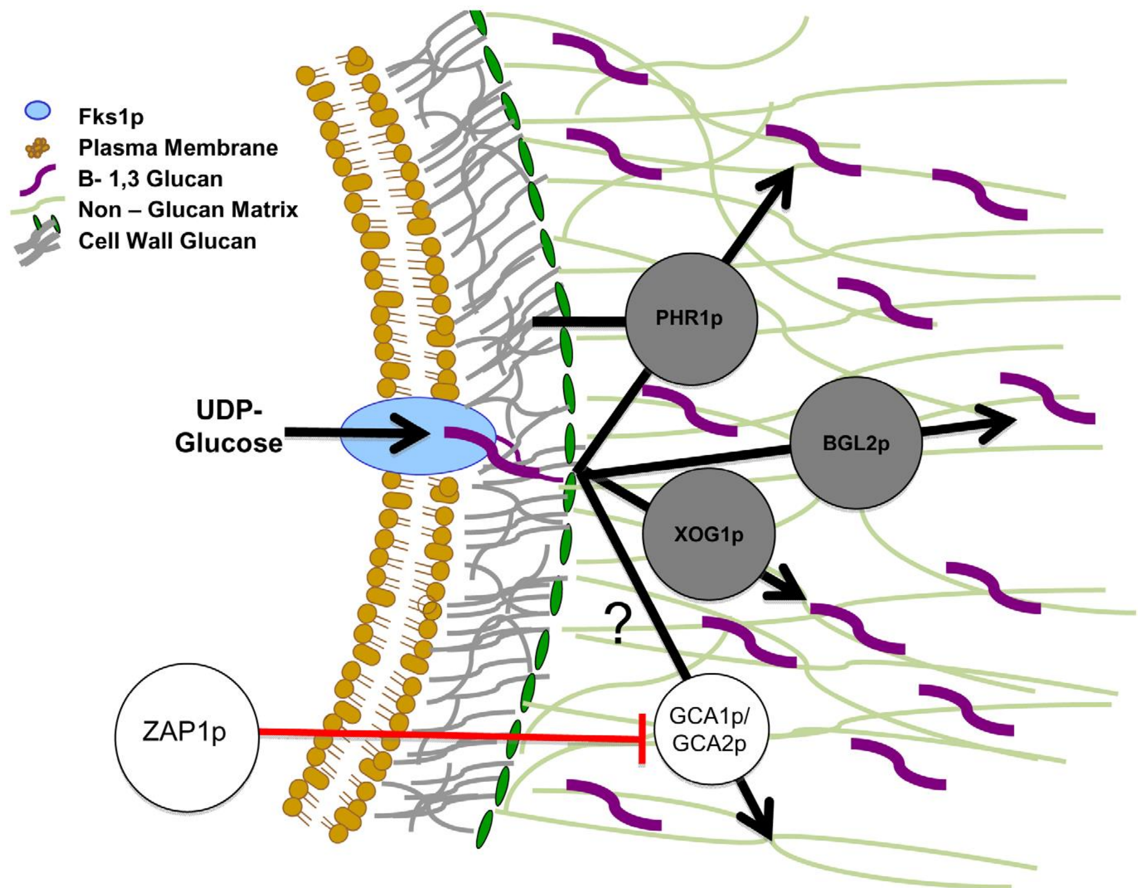

Figura 1.2 – Model for glucan matrix production and delivery to the biofilm matrix. β-1.3-glucan production occurs at the FSK1p cell membrane-bound protein.. The glucan is then modified and incorporated into the cell wall and extracellular matrix. It is proposed that PHR1p, BGL2p and XOG1p act in complementary form and indenpendently from another described pathway form glucan incorporation in the biofilm matrix, the ZAP1p matrix pathway. Matrix glucan are highlited in purple. The cell wall glucan are grey colored The balck arrows represent the pathway taken by glucan from synthase product to the modified sugars that are incorporated into the biofilm matrix. Circles represent modification or production enxymes (adapted from Taff, *et al*.).

BCR1, or Biofilm Cell wall Regulator 1, is a fungal transcription factor required for biofilm formation in *C. albicans* and *C. parapsilosis*. The expression genes that encode some important adhesins as proteins of the *ALS* family or protein from the *HWP* family, are regulated by the *BCR1* gene. Thus gene is involved in the initial adhesion of the cell to the surfaces. Gene deletion studies were performed in both C. albicans and C. parapsilosis strains. As referred previously, *C. parapsilosis* can form a thick layered biofilm. When the *BCR1* gene is deleted, biofilm formed is very sparse and thin. In Candida albicans the same phenomenon was observed. Thus it was safe to conclude that BCR1 gene expression is directly related to the biofilm forming ability of *C. albicans* and *C. parapsilosis* (Ding *et al.*, 2011).

1.7 – Aim

Oral candidosis is an opportunistic infection of the oral cavity caused by the overgrowth of *Candida* species on the oral mucosa. This infection can develop into a systemic and very dangerous disease named candidaemia, being a most common case in immunocompromised patients. *C. albicans* is regarded as the most prevalent species involved in oral candidosis. However in the last three decades, there has been a rise in the prevalence of Non-*Candida albicans Candida* species in clinical sites with *C. parapsilosis*, *C. glabrata* and *C. tropicalis* being identified as other common pathogens. *Candida* species normally exists as commensal organisms in the oral epithelium but some predisposing factors such as diet, age, diabetes among others, can, somehow, turn those commensal microorganisms into pathogenic agents.

The oral cavity is a warm and moist environment, constituted by different structures that create very different habitats in which a large a diverse community of microorganisms lives.. *Candida* species use glucose as main carbon source, although they can survive in the presence of nitrogen-only sources as glutamine. These elements can be provided either from endogenous sources, by saliva, or exogenous sources through food. When food is being processed in the mouth, glucose levels present in the environment can increase up to 10 times. Generally a healthy salivary flow reduces glucose concentration, overtime, but in some diseases, as diabetes, the salivary flow is significantly reduced and glucose basal oral concentration increases.

The ability to form biofilm is one of the most important virulence factors of *Candida* species as it allows the cell to survive in protected communities against starvation, host immunological action and drug therapy. *BCR1* is a transcription factor required for biofilm formation. One of the components of the biofilm structure is β-1,3 glucan, produced by a cell-membrane bound enzyme encoded by the *FKS1* gene. Therefore, the aim of this work was to understand the effect of glucose concentration and presence of the aminoacid L-glutamine on *Candida* species biofilm formation, and its effect in the mentioned biofilm related genes expression.

2 – Materials and Methods

This chapter addresses the techniques and conditions used in this work. Firstly the selected species and strains of Candida are enumerated, as well as, the growth conditions for each assay. Then the analysis techniques and procedures are described, from biofilm biomass quantification, to cell viability assays, structure study and genetic expression.

2.1 - Organisms

A total of 8 strains, a reference strain and an oral isolate of *Candida albicans, Candida parapsilosis, Candida glabrata* and *Candida tropicalis* were used under this work (Table 2.1). The oral isolates (*Candida albicans* 324 LA/94, *Candida parapsilosis* AD, *Candida tropicalis* T2.2 and *Candida glabrata* D1), were obtained from the Biofilm group of the Center of Biological Engineering, and were originally isolated from Clinic of Dentistry, Congregados, Portugal. The reference strains used belonged to the American Type Culture Collection.

Table 2.1 – Species and strains (reference and oral isolate) used under this work.

Specie	Strain
Candida albicans	*Candida albicans* ATCC 90028
	Candida albicans 324 LA/94
Candida parapsilosis	*Candida parapsilosis* ATCC 22019
	Candida parapsilosis AD
Candida glabrata	*Candida glabrata* ATCC 2001
	Candida glabrata D1
Candida tropicalis	*Candida tropicalis* ATCC 750
	Candida tropicalis T 2.2

2.2 - Growth conditions and media

For each assay, strains were subcultured on sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 48h at 37˚C, and then inoculated in sabouraud dextrose broth (SDB) (Merk, Darmstadt, Germany) for 18h at 37˚C under agitation at 120 rpm (overnight). After incubation, cells were harvested by centrifugation at 3000 g for 10 min, and washed twice in PBS (Phosphate-buffered saline 0.1 mM pH 7).

Pellets were ultimately ressuspended in RPMI medium (SIGMA-Aldrich), and cellular concentration adjusted to $1x10^7$ cells/ml using a Neubauer counting chamber. The RPMI medium used changed depending on the condition tested. Ten different RPMI mediums were used. To study the effect of the increase of glucose concentration on biofilm formation by the referred species of *Candida* the mediums used were RPMI with 0.3% of glutamine and increasing concentrations of glucose: 0.2%, 1%,2%,3%,4%,5% and 10% (w/V). To acess the role of glutamine on biofilm formation RPMI medium with 0.3% of glutamine and no glucose and RPMI with no glutamine and with 0.2% and 10%of glucose were also tested.

2.3 - Biofilm formation

The $1x10^7$ cell/ml suspensions previously prepared, were plated into selected wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l' Alleud, Belgium) and incubated at 37˚C on a shaker at 120 rpm for 24 h. The medium was then aspirated and non-adherent cells removed by washing the biofilms twice with PBS.

2.3.1 - Biofilm quantification

Quantification of total biomass was used to access the biofilm forming ability of each strain, at a given condition. Total biomass was quantified by cristal violet (CV) staining. Briefly, after washing with PBS, the biofilms were fixed by pipeting 200µl of methanol into each well. After 15 minutes of contact, the methanol was removed and the wells were allowed to dry at room temperature. Then, 200µl of CV (1% v/v) were added to each well, and the plates incubated for 5 min. The CV was removed, and the wells were gently washed twice with distilled water, and 200 µl of acetic acid was added to each well, to release and dissolve the stain. The absorbance of the obtained solutions were read in triplicate in a microtiter plate reader (Bio-Tek, Synergy HT, Izasa, Lisbon, Portugal) at 570 nm. Three or more (when necessary) assays were performed. The results were presented as abs values/cm^2.

2.3.2 - Biofilm cells viability

Cell viability was determined by counting colony formation units (CFUs) following the ressuspension of biofilm cells. After washing the plate wells with PBS, for each condition, biofilm was detached by scraping with a sterile pippete tip and ressuspended in 200µl of PBS. After biofilm detachment, the PBS from all 3 wells was collected into a single collecting tube and another 100µl of PBS were pipetted into each well, for further scraping. The ressuspended biofilm (900 µl), was vigorously vortexed for 5 min to disrupt the biofilm matrix and serial decimal dilutions were plated onto SDA. The agar plates were incubated for 24 h at 37˚C (overnight), and the colonies enumerated. The results were presented as the total CFU per area unit (Log CFU/ cm^2). Three or more, whenever necessary, independent assays were performed.

2.3.3 - Biofilm structure analysis

To examine the structure of biofilms by scanning electron microscopy 2 ml of the cell suspensions of *Candida albicans* and *Candida parapsilosis* strains($1x10^7$ cell/ml in the several previously referred RPMI mediums) was introduced into 24-wells polystyrene plates (Orange Scientific) and incubated for 24 h at 37° C. Then, the medium was aspirated and non-adherent cells removed by washing the biofilms twice with PBS. Samples were dehydrated with alcohol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min), air dried and kept on a desiccator. The base of the wells were then removed from the plate and mounted onto aluminum stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

2.4 - Gene expression analysis

BCR1 and *FSK1* gene expression was assessed for *Candida albicans* ATCC 90028, *Candida albicans* 324 LA/94, *Candida parapsilosis* ATCC 22019 and *Candida parapsilosis* AD cells from biofilms grown in RPMI medium with 0.2% and 10% glucose and 0% of glutamine, and in RPMI medium with 0.3% of glutamine and 0% of glucose.

2.4.1 - Primer design

Real-time PCR was used to determine the relative expression level of *FSK1* and *BCR1* gene comparatively to the *ACT1* used as a candida housekeeping gene, in RNA transcripts. The primers were designed using Primer 3 web-based software (http://fokker.wi.mit.edu\cgibin\primer3\primer3_www.cgi) and are listed in table 2.2. In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were firstly amplified from *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 genomic DNA. Control *ACT1* primers were also used to detect human recombinant DNA.

Table 2.2 – Primers for Real-time PCR

Species	Sequence (5'→3')	Primer	Target	Product size (bp)
Candida parapsilosis	5'-ATCTGCGTTACCCGCAATAC-3' 5'-ATGTCGTCCACAAACAACGA -3'	Forward Reverse	*ACT1*	228
	5'-CCATTAACCGGGTTGCTATT-3' 5' - GAGTCCGTTATCGCCAATGT-3'	Forward Reverse	*BCR1*	177
	5'- CGACATCCACATGTCCAATC- 3' 5'-CATTGCTGTTGCAACTTTGG-3'	Forward Reverse	*FSK1*	151
Candida albicans	5´-AATGGGTAGGGTGGGAAAAC-3' 5'-AGCCATTTCCATTGATCGTC-3'	Forward Reverse	*ACT1*	168
	5'-GGCTGTCCATGTTGTTGTTG-3' 5'-GAGCACGCATCTATGGCTTA-3'	Forward Reverse	*BCR1*	206
	5'-TGCTTGCCAATGAGAAACTG-3' 5'-ACTGATTTGACCGTTGGT-3'	Forward Reverse	*FSK1*	169

2.4.2 - *Candida* species DNA extraction

For genomic DNA extraction, the *Candida* strains were grown for 18h, at 37˚C, in SDB, following initial subculture and incubation, from stock, in SDA, for 24h at 37˚C. Prior to the DNA extraction, sorbitol buffer, Lysozime buffer and PBS were prepared. The sorbitol buffer is composed by 1.2 M sorbitol, 10 mM $CaCl_2$ 0.1 Tris-HCl (pH 7.5) and 35 mM β-meraptoethanol. The lysozyme buffer was prepared immediately prior to use with the following composition: 20 mg/ml Lysosyme; 20 mM Tris-HCl (pH 8); 2 mM EDTA and 1% of Triton X-100. PBS composition is the same as described above.

Candida cells were harvested by 10 min centrifugation, at 12500g, and the supernatant discarded. Then, 600 µl of sorbitol buffer were added and, after new centrifugation (10 min, 12500g), the pellet was then ressuspended in 1 ml of 1M Sorbitol, 50mM KH_2PO_4, 0.1% β-mercaptoethanol and 0.2 mg/ml Zymolase 100T. After incubation (30 min, 30˚C), the resulting spheroblasts, were collected by centrifugation (10 min, 12500g) and again ressuspended in 0.5 ml of 50 mM of sodium EDTA (pH 8.5) and 2 mg/ml of SDS, and incubated at 70˚C for 30 min. After that, 50µl of potassium acetate was added to the mixture, which was then vortexed and incubated for more 30 min at 0˚C. The supernatant, obtained by centrifugation (12500g, 10 min), was decanted into 1 ml of ethanol and mixed by vortexing. After double centrifugation (12500g, 10 min), the pellet was allowed to dry and then was ressuspended in 0.1 ml of 10 mM tris-chloride-buffer (pH 7.5) and 1mM EDTA (TE buffer containing 10 µg de RNA). After 15 min of incubation at room temperature, 200 µl of 2 – propyl alcohol

was added and the mixture was clarified by centrifugation. The pellet was ressuspended in TE buffer, and stored at -70˚C, for further use.

2.4.3 – Primer specificity assessment

To access primer specificity, a traditional PCR was run for the DNA previously extracted (see section 2.4.2) and for all selected genes (Table 2.1). Briefly, to each gene and each strain of the respective specie, 1µl of DNA sample was collected and 1µl of primer reverse, 1µl of primer forward as well as 12,5µl of Nzytech® Master Mix (2x) were added. Then the following PCR protocol was run: an initialization step at a temperature of 95˚C during 5 min, then the denaturation step at 95˚C during 30 sec, following the annealing step 55˚C for 30 sec and the elongation step at 72˚C during another 30 sec. The last three steps were repeated thirty times. Meanwhile, an 1% agarose gel was prepared in 100 ml of TAE buffer (1x) with 2.5 ml of SYBR® Safe. After PCR conclusion and sample cooling, to selected wells of the agarose gel was added 5µl of sample (PCR amplification product) and 1µl of SYBR® Green. For each strain a negative control was made. Electrophoresis was run on an horizontal lucite slab gel apparatus. The results were accessed using the Nzytech® V DNA molecular weight marker.

2.4.4 - *Candida* species RNA extraction

For RNA extraction, biofilm cells were cultured in the above referred RPMI mediums. Those cells were then collected: the biolfilm cells by washing with PBS and scraping; the planktonic by centrifugation (8000g, 5 min). Cells were disrupted by sonication (velocity 6.5 of intensity during 35 seconds, twice), in RNAase free eppendorfs in presence of glass beads. After centrifugation (14000 rpm, 5 min), the supernatant was collected and an equal volume of etanol 70% (v/v) RNase-free was added. RNA was purified with PureLink RNA Mini Kit®(Invitrogen). Briefly, 500 µl of the mix was transferred for the "Spin Cartridge" column, of the Mini Kit, and then centrifuged at 12000g for 15 sec. This was repeated and then the column was washed with wash buffer I (700 µl).The collection tube, was discarded and replaced by a new. The column was then washed with wash buffer II (500 µl) and the content of the collection tube was discarded (this step repeated one more time). After centrifugation

(12000g, 1 min) at room temperature, the column dried and was transferred for the "recovery tube" supplied with the Mini Kit. About 50 µl of RNase-free water was added, directly on the center of the column, which incubated at room temperature for 2 min, being later centrifuged (12000g, 1 min). The RNA, now diluted in the recovery tube in RNase free-water, was treated with DNase I treatment (Deoxyrybonuclease I, Amplification Grade, Invitrogen), being added to each 10µl of sample 1µl of DNase I, Amplifiation grade. The sample was then inubated for 15 min, at room temperature. In order to deactivate DNase, 1µl of EDTA 25 mM (for each 10µl of sample) was added and the mixture was incubated for 10 min at 65˚C. RNA concentration was determined by optical density measurement, in the reason 260/280 in a nanodrop apparatus (NanoDrop 1000 Spectrophotometer Thermo Scientific®).

2.4.5 - cDNA synthesis

From the RNA obtained, cDNA was produced by adding 8 µl of 5x iscript Reaction Mix, 2µl of iScript reverse transcriptase and 20µl of Nuclease-free water, with a final reaction volume of 40 µl. The synthesis of cDNA was performed at 42˚C for 30 min and the reaction was stopped by heating at 85˚C.

2.4.6 - Quantitative Real-time PCR

In order to optimize the temperature, the cDNA previously obtained was diluted ten times in RNAase free water, and added (4µl), of an 96-wells microtiter plate, in which there already was 10 µl of Supermix (SYBgreen), 4.8 µl H_2O RNAase free, 0.6 µl primer forward and 0.6 µl primer reverse. For each cDNA sample added, an RNA sample, from the same source, was added too, for negative control (NRT). Then a RealTime-PCR protocol was run and the optimal annealing temperature determined, with a difference between the threshold value of cDNA and RNA superior to 10 cycles.

Each reaction mix consisted of the working concentration of Power SYBR® Green master mix (Applies Biosystems), 300 nM of forward and reverse primers, and 1 µl of cDNA, in a final volume of 20 µl. Negative control (water), were included in each run, as well as NRT controls, and genomic DNA decimal dilutions. The relative quantification of *FSK1* and *BCR1* gene expression was performed by ΔCT (threshold cycle) method, using the values for the housekeeping gene to normalize the data. Each

reaction was performed in triplicate and mean values of relative expression analyzed, through the PFAFFL method of quantification, for each gene and condition.

2.5 - Statistical Analysis

Results from the several assays were compared using One-Way analysis of variance (ANOVA) by applying Sidak's multiple comparisons test, using Prism® software (Graphpad Inc., San Diego, California). All tests were performed with a confidence level of 95%.

3 - Results

This chapter presents the obtained results from the worked developed. First total biomass values are presented along with colony formation units counts to understand the effect of glucose variation in the growth medium. The same is done later relatively to the presence/absence of glutamine in the medium. Then, SEM biofilm structure visualization is presented, and lastly the genetic expression results, beginning with the evaluation of primer desing and specificity and ending with the expression results, for different mediums.

3.1 – Assessment of the effect of glucose in *Candida species* biofilm forming ability and cell viability

Figure 3.1, 3.2, 3.3 and 3.4 presents the results of the total biomass quantification through CV staining as well as the respective cell viability values, for *Candida* biofilms formed in RPMI medium with 0.3% of glutamine and supplemented with increasing percentages of glucose 0.2% to 10% of glucose. All values obtained were statistically compared with the values obtained for the condition RPMI supplemented with 0.2% of glucose.

All the studied *Candida* species and strains were able to form biofilms in all conditions, although differences occurred depending on species, strains and conditions tested. In fact, in general, no similarities were found in each condition, between the total biomass values for different strains of the same species. Moreover, no direct correlation was observed concerning the values of total biomass and the CFUs number obtained.

Candida albicans biofilm forming ability was different between the two strains tested (Figure 3). Concerning, *C. albicans* ATCC 90028 the quantity of the biofilm formed does not seem to be dependent of the amount of the glucose present in medium regarding total biomass quantification. It is important to highlight that the number of cultivable cells increased significantly for percentages of glucose of 1% ($p<0.01$), 2%($p<0.0001$) and 5%($p<0.0001$). In the case of *C. albicans* 324 LA/94, the total biomass values remained constant for values of glucose between 0.2% and 1%, but was observed an increase (0.8 times more) in the case of biofilms grown in RPMI supplemented with 2% of glucose ($p<0.001$). Morevoer, for percentages above 3% of glucose, the pattern of biofilm formation decreased significantly for values of absorbance similar to that observed in RPMI supplemented only with 0.2% of glucose. No statistical differences were observed concerning CFUs count for any of the conditions tested.

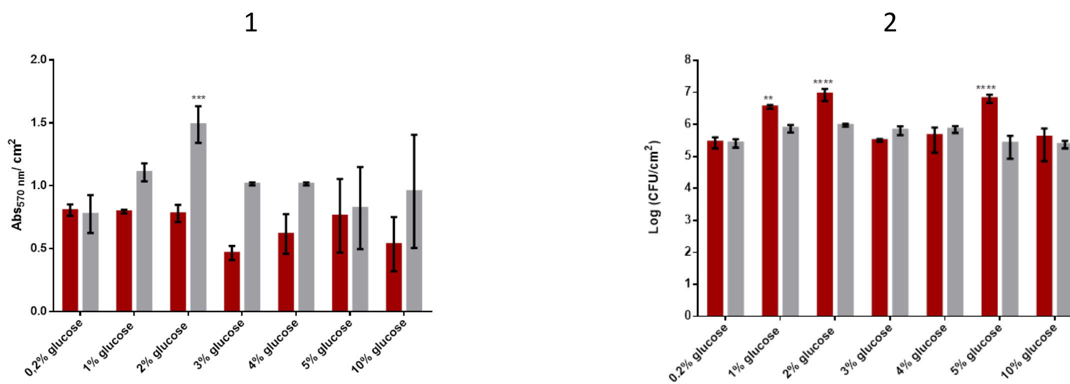

Figure 3.1 – *Candida albicans* biofilm formation ability in presence of different percentages of glucose. Total biomass quantification (1) and number of CFUs (2) values obtained from 24h biofilms at 37º C grown in RPMI medium supplemented with 0.2%, 1%, 2%, 3%, 4%, 5% and 10% of glucose with 0.3% of glutamine. (● reference strain and ● oral isolate). Error bars represent standard deviation. Statically differences obtained when compared with 0.2% of glucose with 0,3% of glutamine (* p<0.1, ** p<0.01, ***p<0.001, ****p<0.0001).

For *C. parapsilosis* strains were observed a clear difference between the oral isolate and the reference strain, with the oral isolate being a more capable biofilm producer (Figure 4). Moreover, It was possible to observe a slight increase in values of total biomass for glucose concentration from 0.2% to 3% in the case of the oral isolate, and a decrease for percentages above 5%. Additionally the biomass values presented a decrease from 3% to 10% of glucose. For *C. parapsilosis* ATCC 22019 was not observed any variability with the increase of the glucose in medium. No correlation was observed between the number of CFUs obtained and the values of total biomass for both *C. parapsilosis* under study.

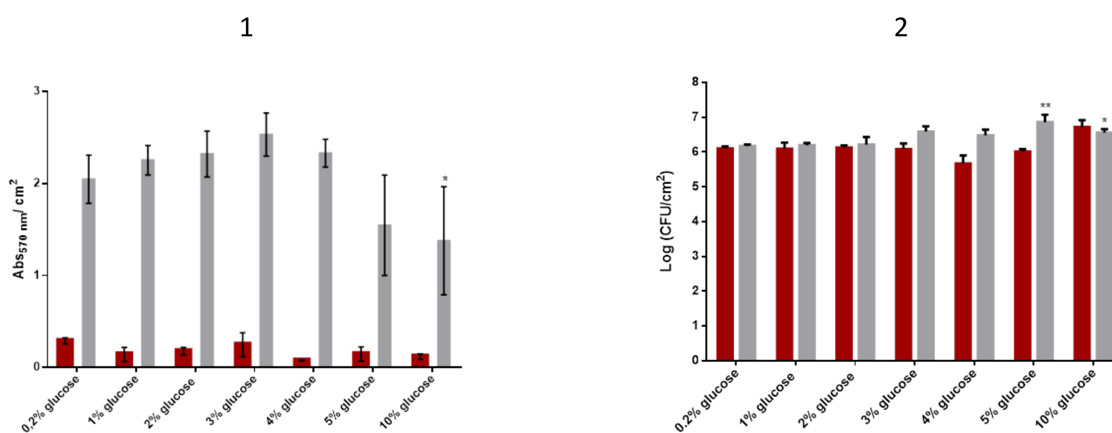

Figure 3.2 – *Candida parapsilosis* biofilm formation ability in presence of different percentages of glucose. Total biomass quantification (1) and number of CFUs (2) values obtained from 24h biofilms at 37º C grown in RPMI medium supplemented with 0.2%, 1%, 2%, 3%, 4%, 5% and 10% of glucose with 0.3% of glutamine. (● reference strain and ● oral isolate). Error bars represent standard deviation. Statically differences obtained when compared with 0.2% of glucose with 0.3% of glutamine (* p<0.1, ** p<0.01, ***p<0.001, ****p<0.0001).

As for the other two species, the values of total biomass were different between the two strains of *C. glabrata* analyzed (figure 4). Again, in the case of the oral isolate, the values of total biomass decreased with the increase of glucose in the medium, specifically for upper of 1%. Concerning the reference strain it was observed an increase in values of total biomass for concentration from 1% to 4% of glucose in the medium it is important to stress that for values above 5% a decrease in capability to form biofilm was notorious. On the other hand, the CFUs values were more constant without relevant oscillations observed.

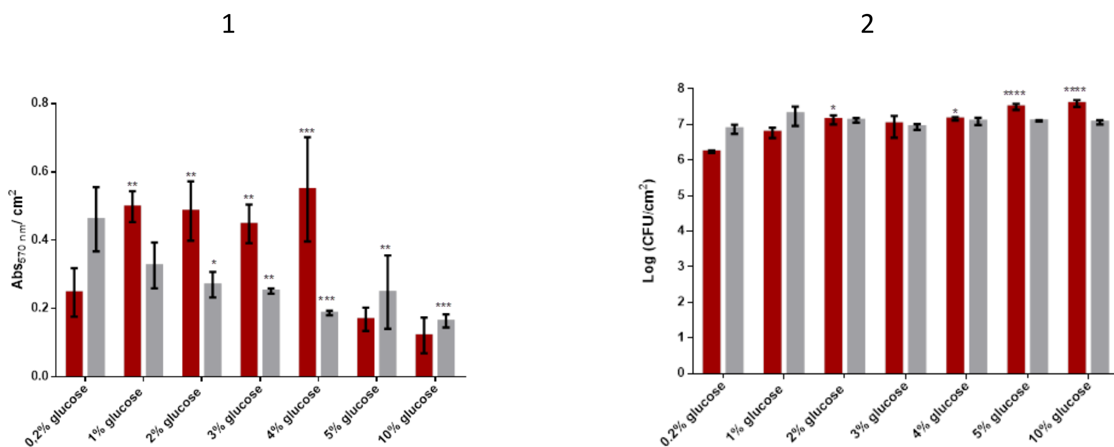

Figure 3.3 – *Candida glabrata* biofilm formation ability in presence of different percentages of glucose. Total biomass quantification (1) and number of CFUs (2) values obtained from 24h biofilms at 37º C grown in RPMI medium supplemented with 0.2%, 1%, 2%, 3%, 4%, 5% and 10% of glucose with 0.3% of glutamine. (● reference strain and ● oral isolate). Error bars represent standard deviation. Statically differences obtained when compared with 0.2% of glucose with 0.3% of glutamine (* p<0.1, ** p<0.01, ***p<0.001, ****p<0.0001).

Candida tropicalis reference strain presented less ability to form biofilm than the oral isolate (figure 6) The values of total biomass produced in medium with 3% (p<0.01), 4% (p<0.001), 5% (p<0.1) and 10% (p<0.01) of glucose were significantly lower than the values obtained for medium with 0.2% of glucose, in the case of *C. tropicalis* reference strain. Moreover analyzing the pattern of the *C. tropicalis* reference strain no significant changes were observed. On the other hand, the number of CFUs remained homogenous for all conditions tested and both strains, with exception of the 5% (p<0.001) in both strains and 10% of the glucose (p<0.0001) only in case of the ATCC 750 strain.

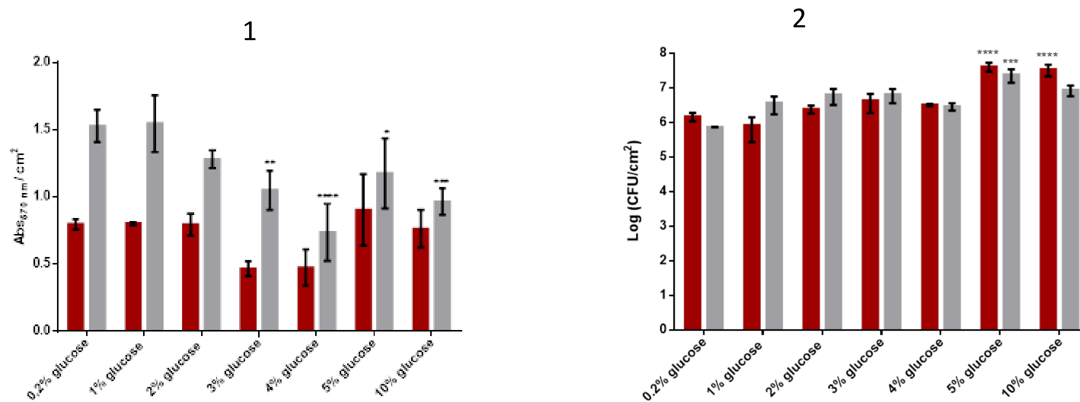

Figure 3.4 – *Candida tropicalis* biofilm formation ability in presence of different percentages of glucose. Total biomass quantification (1) and number of CFUs (2) values obtained from 24h biofilms at 37º C grown in RPMI medium supplemented with 0.2%, 1%, 2%, 3%, 4%, 5% and 10% of glucose with 0.3% of glutamine. (● reference strain and ● oral isolate). Error bars represent standard deviation. Statically differences obtained when compared with 0.2% of glucose with 0,3% of glutamine (* p<0.1, ** p<0.01, ***p<0.001, ****p<0.0001).

3.2 – Assessment of the effect of the glutamine on *Candida albicans* and *Candida parapsilosis* biofilm formation and in cell viability.

Candida albicans and *Candida parapsilosis* strains were grown with 0.2% and 10% of glucose in RPMI mediums with and without 0.3% of glutamine, as well as in RPMI medium supplemented with 0.3% glutamine and without of glucose (Figure 3.5 and 3.6).

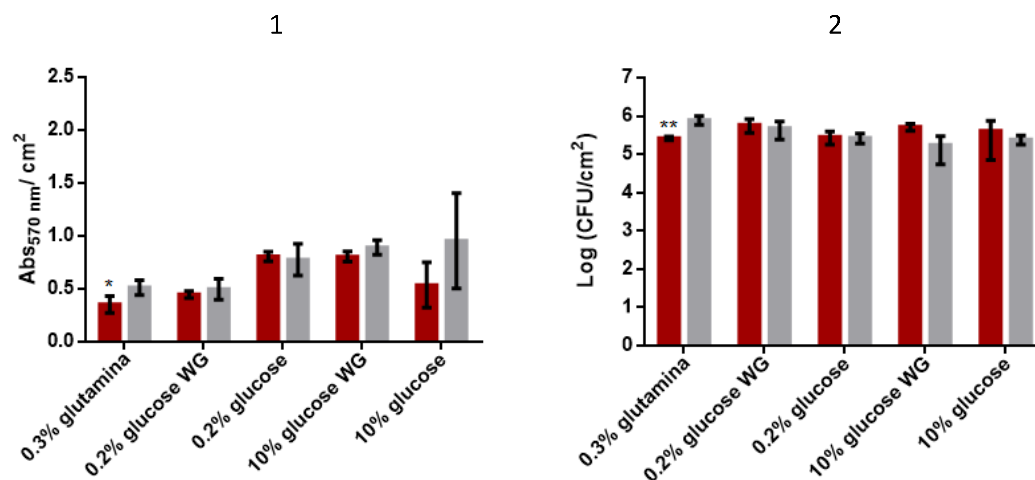

Figure 3.5 – Influence of glutamine in *Candida albicans* biofilm formation ability. (1) Total biomass quantification (1) and number of CFUs (2) values obtained from 24h biofilms at 37º C grown in RPMI medium supplemented with 0.3% of glutamine without glucose and 0.2% and 10% of glucose in presence(glucose) and absence (glucose SG) of 0.3% of glutamine. (● reference strain and ● oral isolate). Error bars represent standard deviation. Statically differences obtained when compared with 0.2% of glucose with 0.3% of glutamine (* p<0.1, ** p<0.01, ***p<0.001, ****p<0.0001).

Figure 3.5 presents the results obtained concerning the effect of glutamine in biofilm formation of *C. albicans* strains. As it was possible to observe, the reference strain presented lower capability to form biofilm in only in presence of glutamine and in total absence of glucose, as could be confirmed for the values of CV ($p<0.1$) and CFUs ($p<0.01$) obtained. In case of the oral isolate were not observed significant difference between all conditions tested.

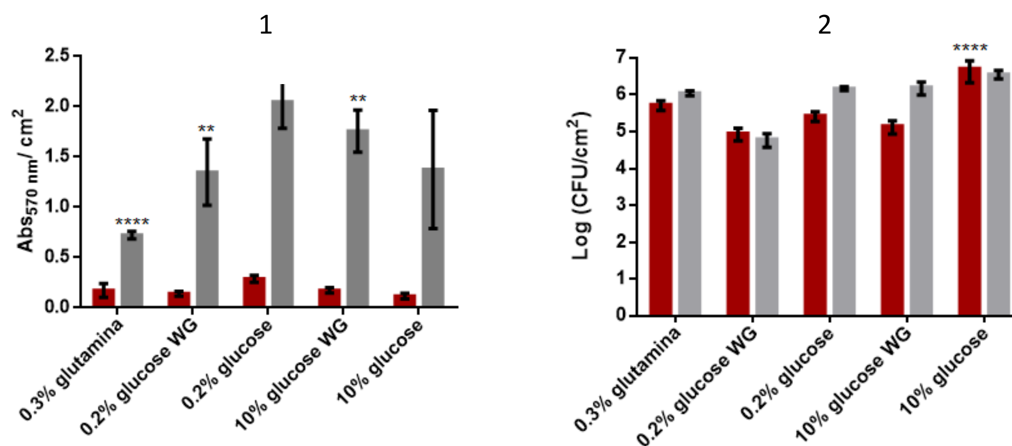

Figure 3.6 – Influence of glutamine in *Candida parapsilosis* biofilm formation ability. (1) Total biomass quantification (1) and number of CFUs (2) values obtained from 24h biofilms at 37º C grown in RPMI medium supplemented with 0.3% of glutamine without glucose and 0.2% and 10% of glucose in presence (glucose) and absence (glucose SG) of 0.3% of glutamine. (● reference strain and ● oral isolate). Error bars represent standard deviation. Statically differences obtained when compared with 0.2% of glucose with 0.3% of glutamine (* p<0.1, ** p<0.01, ***p<0.001, ****p<0.0001).

Candida parapsilosis presented a significant difference between the two strains tested contrarily to *C. albicans* strains. In fact, as in the other assays performed, *Candida parapsilosis* ATCC 22019 formed less biofilm than the oral isolate (figure 3.6). Specifically, concerning the reference strain were not observed any statistically significant difference, in terms of total biomass. However, it was possible to observe an increase in the number of CFUs in the presence of 10% of glucose with 0.3% of glutamine ($p<0.0001$). The oral isolate formed also lower quantity of biofilm in RPMI supplemented with 0.2% and 10% glucose ($p<0.01$), without glutamine, and with only 0.3% glutamine, without glucose ($p<0.0001$). In terms of CFUs, there was no observed statistically significant difference between any conditions tested.

Once again, it was noticeable a discrepancy, through the several conditions, between *Candida* species and strains biofilm forming ability concerning biofilm

forming ability. Moreover, the glucose seems to be a principal role in biofilm than the glutamine, once the biofilms formed in absence of glucose and in the presence of glutamine presented in general lower values of total biomass.

3.3 –Influence of glucose and glutamine in *Candida albicans* and *Candida parapsilosis* biofilms structure.

The biofilm structure and the cells morphology of the *Candida albicans* and *Candida parapsilosis* strains were analyzed through SEM. For that, 24h biofilms of *C. albicans* ATCC 90028, *C. albicans* 324 LA/94, *C. parapsilosis* ATCC 22019 and *C.parapsilosis* AD, were grown in 0.3% glutamine medium RPMI medium without glucose and in RPMI medium supplemented with 2% and 10% of glucose without glutamine (Figure 9).

Candida albicans ATCC 90028 biofilms consists of a network of cells of a variety of morphologies(figure 9 A). Biofilms formed in RPMI only supplemented with glutamine, presents as a thin, shallow layer of yeast and filamentous forms (figure 9 A - 1). Moreover, when grown in glucose RPMI medium, the biofilm showed a more dense structure, with thicker and longer hyphal forms. The increase for 10% of glucose in medium, results in an increased amount of hyphal filamentous forms in a more dense network (figure 3.7 A - 3).

Candida albicans 324 LA/94 biofilms grown in RPMI glutamine free of glucose, presented more quantity of ECM covering layers of yeast and hyphal forms, relatively to the other strains. As the glucose in the medium increases (from 0.2% to 10%) a higher number of hyphal forms appears in the biofilms.

C. parapsilosis ATCC 22019 biofilm structure (Figure 3.7 C and D) formed micro communities of yeasts anchored to surface. However, it is important to address an increase in size of the micro communities with the increase of the percentages of glucose in medium. *C. parapsilosis* AD, in the absence of glucose, forms only agglomerates of yeast cells. Interestingly, it was observed that in the presence of glucose, the size of this clusters increase and pseudohyphal structures emerge (see arrows (Figure 3.7 D - 3). An alteration on RPMI composition is able to influence the structure of *C. parapsilosis* and *C. albicans* biofilms as well as its cell morphologies.

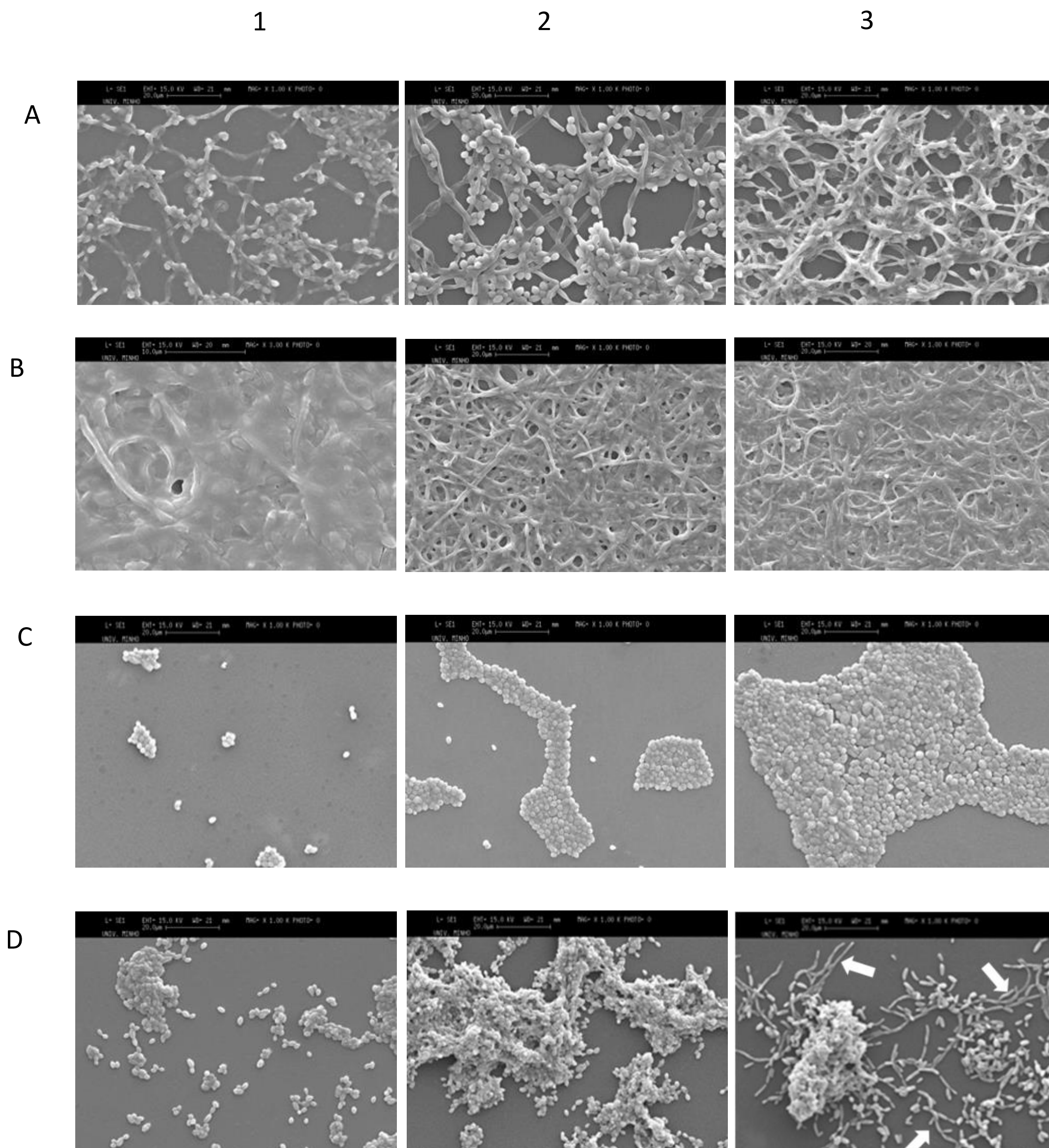

Figure 3.7 – Scanning electron microscopy of *Candida* species. biofilms after 24 h. a) *Candida albicans* ATCC 90028, b) *Candidas albicans* 324 LA/94, c) *Candidas parapsilosis* ATCC 22019 and d) *Candidas parapsilosis* AD in RPMI medium 1) 0.3% of glutamine and 0% glucose, 2) 0.2% of glucose and 3) 10% of glucose. The bar in the images corresponds to 20 µm. Magnification 1000x.

3.4 – Assessment of the expression level of *BCR1* and *FSK1* gene in biofilm cells in presence of glutamine or glucose.

To access the genetic effects of glucose percentage and presence/absence of glutamine in the growth medium, the relative expression levels of two *Candida* biofilm-related genes was determined. The studied genes were: *FSK1* gene, who codifies a cell-membrane enzyme that produces β-1,3 glucan, from glucose, which will be incorporated into the biofilm matrix; and the *BCR1* which codifies a regulator

protein important for encoding biofilm matrix proteins. The *ACT1* gene, that is constitutively expressed, codifies for the actin and for that was selected as the housekeeping gene (the normalize gene).

Figure 3.8, presents the results of the traditional PCR with genomic DNA extracted from the 4 strains relatively to the 3 genes under study. This was performed in order to access the correct design of the primers and its specificity (Table 2.2).

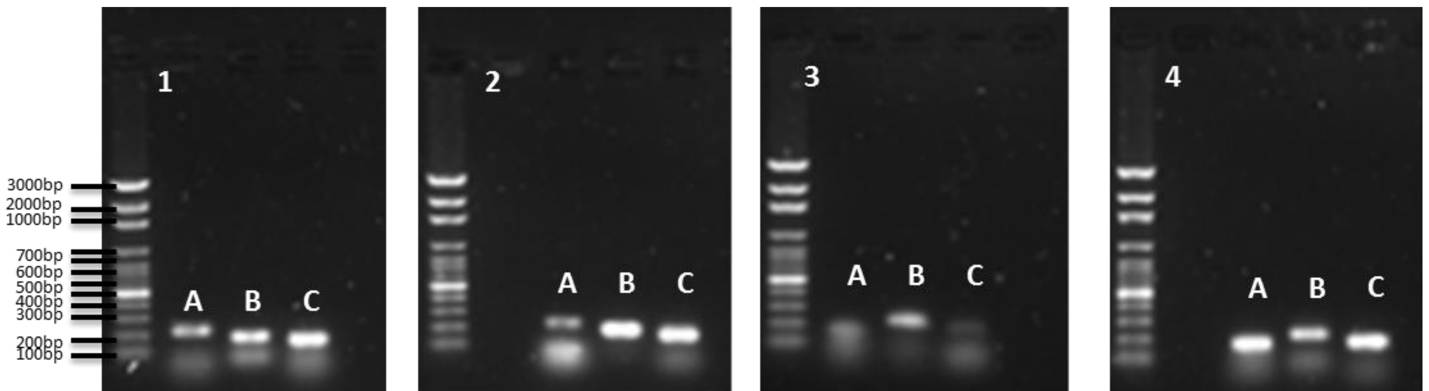

Figure 3.8 – Results of primer specificity , in DNA gel 1% of agarose, relatively to the genes A) *ACT1*, B) *FKS1* and C) *BCR1* for the strains: 1) *Candida albicans* ATCC 90028, 2)*Candida albicans* 324 LA/94, 3)*Candida parapsilosis* ATCC 20019 and D) *Candida parapsilosis* AD.

As it is possible to observe, all primers were well designed, once that all the products of amplification presented the expected size. Namely, for *C. parapsilosis* with a product of amplification for *BCR1* and *FKS1* between the 100 bp and 200 bp marker bands, with the correct size of 177 bp and 151 bp respectively. The *ACT1* gene amplification product appeared above the marker of 200 bp, also in accordance with the dimension of the target expected, 228 bp. Regarding *C. albicans*, the gel showed that *ACT1* and *FKS1* amplification product appeared at the same level, between the marker's bands of 100 bp and 200 bp, in accordance with the target dimension of 168 bp and 169 bp, respectively. The BCR1 amplification product appears slightly above the 200 bp marker's band, also in accordance with the target dimension expected of 206 bp.

The work followed with the genes expression studies by quantitative real time PCR for the *FKS1* and *BCR1*. For that, all four *Candida* strains biofilms grown during 24h in RPMI supplemented with 0.2% of glucose, 10% of glucose (both with 0% of

glutamine) and in RPMI medium supplemented with 0.3% of glutamine but without glucose. The expression of *FKS1* and *BCR1* gene levels values were normalized by the values of the expression obtained for the housekeeping selected gene, *ACT1*.

Figure 3.9 and 3.10 presents *BCR1* and *FSK1* gene expression levels for all conditions and strains under studied. It was possible to observe an up-regulation of *BCR1* gene from 0.2% to 10% of glucose in both *C. albicans* strains. Additionally, it was also observed that the oral isolate expressed in higher intensity the *BCR1* than the reference strain in both conditions (0.2% and 10% glucose). The levels of expression of *BCR1* were higher in the presence of glutamin (p<0.1) than for biofilm grown in RPMI supplemented only with 0.2% of glucose.

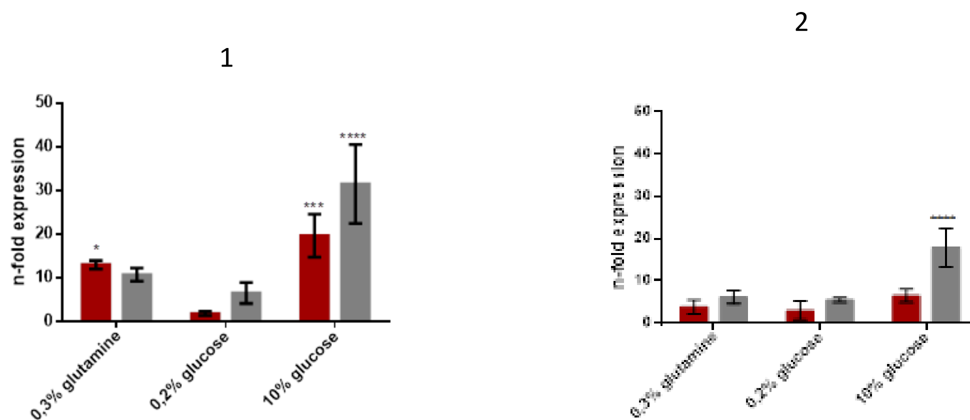

Figure 3.9 – Results of BCR1 gene expression for (1) Candida albicans and (2) Candida parapsilosis.(● stands for reference strain and ● stands for oral isolate). Error bars represent standard deviation. Statically significant differences obtained when compared with the expression levels for 0.2% of glucose RPMI medium (* p<0.1, ** p<0.01, ***p<0.001, ****p<0.0001).

The levels of *BCR1* expression concerning *C. parapsilosis* were statistically different from other conditions in case of 10% of glucose(2 fold over) for the clinical isolate (p<0.0001). No statistical differences in the expression levels of this gene for *C. parapsilosis* were detected. Thus, it is possible to assume that BCR1 gene expression is were glucose concentration dependent for *C. parapsilosis* e *C. albicans* species, but its expression does not depend on the presence of glucose in the medium, since its substitution by glutamine, does not cause a decrease in the expression of this gene.

The FKS1 levels of expression for both species are presented in figure 3.10. Downregulation of the expression of *FKS1* was observed for *C. albicans* grown in RPMI medium supplemented with 0.3% of glutamine for both strains (p<0.01 and p<0.1,

respectively for reference and clinical isolate). Interestingly it was to observe an over-expression of *FKS1* in biofilms formed by *C. albicans* ATCC (P<0.01) and C. albicans oral isolate (p<0.0001) in presence of 10% of glucose.

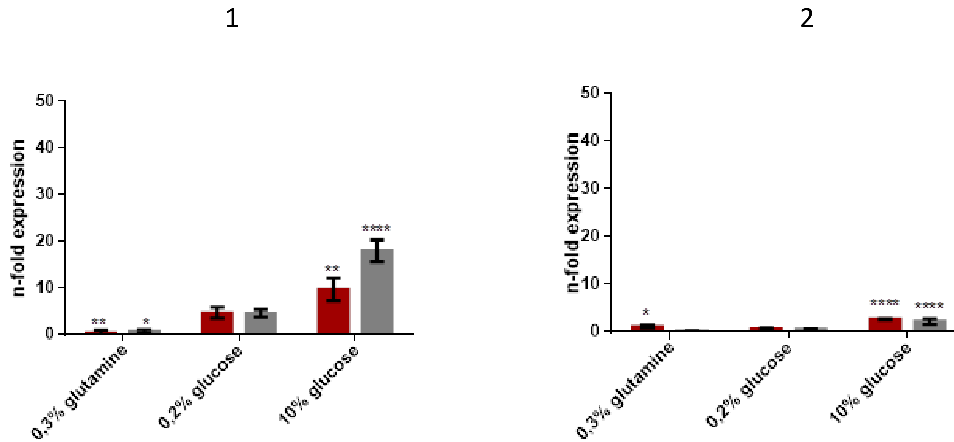

Figure 3.10 – Results of *FSK1* gene expression for (1)Candida albicans and (2)Candida parapsilosis normalized by the *ACT1* housekeeping gene expression for each strain (● stands for reference strain and ● stands for oral isolate). Error bars represent standard deviation. Statiscally significant differences obtained when compared with the expression levels for 0.2% of glucose RPMI medium (* p<0.1, ** p<0.01, ***p<0.001, ****p<0.0001).

An over-expression of *FKS1* gene was also detected in the cells from 10% of glucose biofilms formed by both *C. parapsilosis* strains (p<0.0001). Curiously, it was also observed in *C. parapsilosis* ATCC 22019 a slight up-regulation for biofilm grown in presence of 0.3% of glutamine without glucose (p<0.1).

The expression levels of both gene was strain, specie and condition tested dependent. For *C. albicans*, it seems to strictly glucose dependent. For *C. parapsilosis* the expression increases as the glucose concentration rise from 0.2% to 10% of glucose, but the expression does not decrease when glucose is replaced by glutamine.

4 - Discussion

This chapter consists on a thorough analysis on the results presented in the previous section. The values are compared with the theoretical expected results and possible discrepancies are addressed, as well as explanatory hypothesis

Biofilm formation is an important virulence factor of *Candida* species, contributing to its pathogenicity and associated with high morbidity rates in cases of oral candidosis (Fanning *et al.*, 2012; Yanq, 2003). It confers *Candida* species significant resistance to both antifungal agents and host immune response, allowing the cells to resist to several environmental stress factors, such as host defense mechanism and competitive pressure from other microorganisms. In biofilms, cells are aggregated in communities embedded within a complex ECM, which limits the penetration of substances hindering them to reach the cells, which is the case of antifungal agents.(Silva *et al*., 2011) Biofilm formation ability of *Candida* species is dependent upon several environmental factors including the available carbon or nitrogen source, and respective concentration.(Hawser *et al*., 1994) In the oral environment, the concentration of the glucose levels in saliva can vary greatly from values, in terms of mass per volume, around 1.38% to values around 8%, in terms of weight per volume, or higher depending on food intake, oral higiene, salivary flow and diabetes.(Abikshyeet *et al*., 2012; Panchbhai, 2012) Thus, the aim of this work was to sutdy the influence of glucose and glutamine in *Candida* species biofilm formation ability.

4.1 - Assessment of the effect of glucose in *Candida species* biofilm forming ability and cell viability

In this study, the biofilm formation ability of several *Candida* species (Table 2.1) in media with different concentrations of glucose was evaluated and the results (Figures 3.1, 3.2, 3.3 and 3.4) showed that all strains were able to form biofilm in all studied conditions, although at different amounts depending on species, strain and condition. These results are in accordance with previous studies that showed that the ability of the *Candida* species and strains in forming biofilms *in vitro* is strain and species dependent (Negri *et al*., 2012; Negri *et al*., 2011; Silva *et al*., 2012; Silva *et al*., 2009; Silva *et al*., 2011).

As previously described by Silva *et al. Candida parapsilosis* AD, was the best biofilm forming strain, which biofilms exhibited the greates ability to retain crystal violet for all studied conditions (figure 3.2). Curiously, *C. parapsilosis* ATCC 22019, had

a very low capacity of forming biofilm, being almost equivalent to that of *C. glabrata* (figure 3.3). Glucose percentage variation had little effect on the biofilm formation ability for the reference strain; though a slight and not significant successive increase in the amount of biofilm formed can be noticed as the glucose levels increase from 0.2% to 3% for the oral isolate (figure 3.2). Therefore, biofilm formation appears to be glucose dependent only for the *C. parapsilosis* oral isolate, and considering the decrease in the amount of biofilm biomass registered for glucose percentages above 5%, this seems to be the maximum peak concentration supported by this strain before stress is induced. These results are in accordance with the previously reported tendency of *C. parapsilosis* isolates being normally isolated from glucose-rich environments such as parental nutrition devices, in clinical cases (Trofa *et al*., 2008).

In the case of *C. albicans*, the amount of biofilm formed by the reference strain seemed to slightly vary across some conditions, although no direct relation can be made with the amount of glucose present in the formation medium. The oral isolate, on the other hand, formed more biofilm as the glucose percentage increased to 2% and decreasing above that value. Thus, it seems that also for *C. albicans*, the oral isolate biofilm formation capacity is more glucose dependent than the reference strain. This is corroborated by earlier studies that showed a great preference of oral isolates of *C. albicans* for glucose relatively to alternative carbon sources (Jin *et al.*, 2004), and also a strong dependence of biofilm formation to carbon source concentration (Kuhn *et al.*, 2002).

Regarding *C. glabrata*, these species formed the lowest amounts of biofilm comparatively to other species, as showed by Silva, *et al.*(figure 3.3) (Silva et al., 2009). The reference strain produced more biofilm in the medium as the concentration of glucose increased (from 0.2 to 4%), while for the oral isolate, the amount of biofilm produced decreased significantly above 1% until 10% of glucose. In opposition to what was observed for *C. albicans* and *C. parapsilosis*, the *C. glabrata* reference strain biofilm forming ability seems to be more glucose dependent than the oral isolate, which in fact do not tolerates high glucose percentages. This results are in accordance with previous reports in which *C. glabrata* isolates formed biofilm preferentially in mediums with low concentrations of glucose over mediums with higher concentrations of other carbon sources (Hawser *et al*., 1994).

Candida tropicalis oral isolate biofilm formation capacity presented the same behavior as the *C. glabrata* oral isolate, for glucose percentages above 3% to 10% of glucose. From 0.2 % to 2%, it remained relatively constant, being 1.5 times greater than the values obtained for the reference strain. The peak of the glucose value seems to be at 2% of glucose concentration in the medium. Before that start forming less biofilms as the glucose percentage increases, but its biofilm formation capacity does not seem to be glucose dependent. The amount of biofilm formed by the reference strain was relatively homogeneous through all conditions and so, biofilm formation capacity by this strain is not dependent on the amount of glucose present in the medium.

The variation of glucose concentration in the biofilm formation medium was also tested in terms of biofilm cell viability, after 24h. For all the conditions, there was not a direct relation between the amount of biofilm formed and the number of viable cells. In general, the differences in the amount of formed biofilm between strains of the same species did not correspond to a similar difference in the number of CFUs, for each condition. In figure 3.2, is possible to observe that the amount of CFUs is similar between the two strains of *C. parapsilosis*, despite of the great difference registered in terms of biofilm forming ability. The same is valid for several conditions in the other species and strains under studied (figures 3.1, 3.3 and 3.4). Glucose variation effect in biofilm also did not correspond in a similar variation in terms of number of viable cells in case of *C. albicans*. While the oral isolate amount of formed biofilm increased when glucose varies from 0.2% to 2%, the number of viable cells remained constant. In contrast, the reference strain amount of formed biofilm remained constant through that condition, but the number of viable cells significantly increased from 0.2% to 2% of glucose (figure 3.1). Similar observations were made in the case of the other strains (figures 3.3 and 3.4), and so, is reasonable to assume that the number of viable cells in the medium does not correlate with the amount of biofilm biomass measured by crystal violet. Indeed, crystal violet uptake on cell morphology and size and this stain can also be entrapped in the biofilm matrix, therefore it cannot be used to access the number of cells in the biofilm but, roughly, the amount of total biomass.

4.2 - Assessment of the effect of the glutamine on *Candida albicans* and *Candida parapsilosis* biofilm formation and in cell viability

The aminoacid L-glutamine can act as a nitrogen source for *Candida* species allowing them to survive through the production of essential proteins, in the absence of a carbon source. However, due to the high amounts of carbohydrates present in the biofilm, the question is raised about the capacity of biofilm formation by *Candida* in mediums without glucose and the effect that glutamine has in the presence of glucose (Holmes *et al.*, 1989). Considering the previous results discussed in section 5.1, only for *C. parapsilosis* and *C. albicans* oral isolate strains the total biofilm biomass values increased, with the increase of the percentage of glucose in the growth medium. Thus, is reasonable to say that this two species oral isolate strains were the most glucose dependent in terms of biofilm formation ability, in regards to all the studied strains. For that reason, the next described studies and results will focus only on these two species.

From the results present in figure 3.5 for *C. albicans,* it seems that the presence or absence of glutamine in mediums supplemented either with low or high amounts of glucose is insignificant for biofilm formation or cell viability. The biofilm formation capacity for this species for the medium supplemented only with 0.3% of glutamine, was lower in the case of the reference strain and similarly in the case of the oral isolate, when compared to the amount of formed biofilm for the medium with 0.2% of glucose and 0.3% of glutamine. In terms of CFUs, the same was observed, being the number of viable cells of the reference strain also lower, for the medium with only glutamine, when compared to those from the reference medium. Thus it seems that the oral isolate can adapt well to the total absence of glucose from the medium, while the reference strain cannot. These results are in opposition with previous reports that revealed that glucose is the carbon source that allowed to *C. albicans* to form biofilm (Hawser *et al*., 1994; Jin *et al*., 2004).

For *C. parapsilosis* (figure 3.6), the great difference between the two strains in terms of biofilm formation are still valid for all the conditions. In the mediums with only glucose and without glutamine, the oral isolate formed statistically less biofilm when compared to the amount of biofilm formed for the medium with 0.2% of glucose and 0.3% of glutamine. However, the difference was greater for the medium with only

glutamine. The oral isolate cell viability was not affected by the absence/presence of glucose or glutamine in the medium. The same was observed for the reference strain biofilm formation capacity and biofilm cell viability. Therefore, *C. parapsilosis* oral isolate seems to be more affected by the alterations in terms of energy source in both strains.

4.3 – Influence of glucose and glutamine in *Candida albicans* and *Candida parapsilosis* biofilms structure.

SEM processing is a method that has an inherent destructive capacity , but can provide useful information in both biofilm structure and cell morphology (Silva *et al*., 2009). In the present work, SEM processing revealed structural differences in 24h biofilms of *C. albicans* ATCC 90028, *C. albicans* 324 LA/94, *C. parapsilosis* ATCC 22019 and *C. parapsilosis* AD formed in RPMI mediums with 0.3% glutamine and without glucose or with 0.2% or 10% of glucose without glutamine, depending on specie, strain and condition.

Biofilm structure and cell morphology are dependent on several environmental factors including growth conditions.(Hawser *et al*., 1994) Biofilms of *Candida albicans* (figure 9 A and B) are composed by yeast and filamentous forms, in accordance with earlier reports (Brown, 2002; Gow, 2002). The hyphal forms are larger as the glucose percentage increases, as well as the biofilm overall structure complexity. In *C. albicans* ATCC 90028 (figure 9 A) the biofilms grows structurally as the glucose concentration in the medium increases, from 0.2% of glucose to 10% of glucose, with the appearance of more interconnected and thick hyphal forms. The structure of *C. albicans* 324 LA/94 biofilms presents high amounts of ECM covering layers of yeast and filamentous forms. The presence of glucose and its percentage increase in the medium leads to an increase in the number of hyphal forms and respective size resulting in a more complex structure.

Regarding *C. parapsilosis*, the reference strain biofilms showed (figure 9 C), microcommunities of yeast cells anchored to the surface, which increased in size as the percentage of glucose in the medium increased. The oral isolate biofilms consisted of agglomerates of yeast cells in RPMI medium with only glutamine, and in the presence

of glucose not only the cells and clusters increased in size but also pseudohyphal structures emerged.

In general, except for *C. parapsilosis* reference strain (figure 3.6), the results for viable cells did not increased with the increase of glucose percentage, contradicting the increase of biofilm density in observed through SEM. As this raised was followed also for a rise in the number of filamentous forms for both species, this discrepancy may be due to the nature of the cell viability assay method, that cannot access correctly the number of clustered cells or hyphal forms.

4.4 - Assessment of the expression level of *BCR1* and *FSK1* gene in biofilm cells in presence of glutamine or glucose

In order to access in more detail the effect on biofilm formation of the variation of glucose percentage in the growth medium as well as the presence or absence of glutamine, the expression levels of two Candida genes related with biofilm formation were assessed. Before being able to form biofilms, *Candida* yeasts must first adhere to the surface. Several cell wall glycoproteins and polysscharides are involved in this process such as proteins encoded by aglutinin-like sequence gene family or Hyphal cell wall-specific protein gene (Chauhan *et al*., 2002; Hoyer, 2001; Silva *et al*., 2011). The expression of this genes are regulated by the *BCR1* gene, a fungal transcriptional factor. Studies have proved a direct relationship between the expression of this gene and biofilm formation.(Ding *et al.*, 2011) *BCR1* expression for strains *C.albicans* was higher in both strains for the medium with 10% of glucose, comparatively to the medium with 0.2% of glucose, as well as in the medium supplemented only with 0.3% of glutamine, especially in the case of the reference strain. Given that no statistical differences were observed in terms of biofilm biomass values for those conditions (figure 3.5), was surprising to observe such difference in terms of gene expression. In case of *C. parapsilosis*, *BCR1* expression was similar between the two strains, being significantly lower in the medium without glucose and significantly higher in the medium an increased glucose concentration (figure 3.10). Although the gene expression obtained was expected for the RPMI medium with only glutamine, in which both strains formed less biofilm, the increase in expression for the medium with 10% of glucose and the similarity in the expression values between strains were

unexpected. (figure 3.6 and figure 3.7 C). Therefore, and given the CFUs results in figure 3.6, it seems the great differences obtained in the absorbance values of crystal violet between the two strains for all studied conditions was due to an higher capacity in ECM production of the oral isolate.

An active element of the biofilm matrix, responsible for drugs capture is β-1,3 glucan, which is synthesized from UDP-D-glucose through the β-1,3 glucan synthase, a membrane-bound enzyme, constituted by two subunits, one of them being genetically encoded by the *FKS1* gene. (Taff *et al*., 2012) In *Candida albicans FSK1* gene expression was significantly lower in the medium without glucose and significantly higher in the medium with an increased glucose percentage, as expected. For *C. parapsilosis* the gene expression was also higher lower in the mediums with 10% of glucose, being similar between the other conditions. Overall, *FKS1* expression values in *C. albicans* were higher than the expression values in *C. parapsilosis*, through all conditions. These results hint that *C. albicans* biofilms may have more β-1,3 glucan included in its cell wall and biofilm matrix that *C. parapsilosis*, considering the fact that the oral isolate of this species was the one that formed more biofilm, of all studied strains. These results are in accordance with what was observed through SEM images of the biofilms. As described in the previous section, *C. albicans* oral isolate presented more extracellular matrix in its biofilm than any other strain.

5 – Concluding remarks

This chapter presents the major conclusion taken from the obtained results and posterior discussion, addressing all aspects of the developed worked.

Oral candidosis is a common opportunistic infection of the oral cavity caused by the overgrowth of *Candida* species that can develop into a deadly systemic infection known as candidaemia. The high pathogenicity of *Candida* species is related to biofilm formation. An high carbohydrate diet, an impaired salivary function, diabetes, are some factors related to incidence of candidosis and can lead to a rise in the glucose concentration in the mouth. Therefore, the aim of this study was to access the effect of glucose and glutamine in biofilms formation by *Candida* species.

All the studied *Candida* strains formed biofilms in all the selected conditions, and the glucose concentration variation as well as the presence/absence of glutamine in the medium had different effects on the amount of biofilm formed, although depending on specie and strain. *Candida albicans* and *Candida parapsilosis* oral isolates were able to augment their biofilm formation abilities in the presence of increasing concentrations of glucose until a maximum value level of glucose, above which, the amount of produced biofilm started to decrease. The biofilm formation ability of the reference strains of both species remained indifferent to the glucose percentage variation in the medium. Both *Candida glabrata* strains were less able to form biofilms in all conditions. Thus, although an increase was noticed in the amount of biofilm produced for the reference strain, overall the amount of biofilm produced was not significant when compared to the other strains The biofilm formation ability of the *Candida tropicalis* reference strain was indifferent to glucose concentration increase and the oral isolate formed more biofilm in lower glucose values, being less able to do it when the glucose concentration increased. Therefore, only *C. albicans* and *C. parapsilosis* strains were tested for the effect of glutamine in the growth medium. Moreover, the huge differences observed in terms of biofilm forming ability did not correlate with the values obtained for the number of cultivable cell, for different conditions, strains and species, hinting at big differences in terms of ECM production ability.

Glutamine absence in the growth medium, for mediums supplemented with glucose, had a significant effect for *Candida parapsilosis* oral isolate, which formed less biofilm. In regards to the other strains, their biofilm formation ability was not disturbed by the lack or presence of glutamine when glucose was present. Substitution of glucose by glutamine, led to a decrease in biofilm formation only for *C. albicans*

reference strain and *C. parapsilosis* oral isolate, hinting at physiological differences of strains of the same species.

SEM images revealed structural differences between biofilms of strains and species of *C. albicans* and *C. parapsilosis*. In general, *C. albicans* 324 LA/94 biofilms presented a more extracellular matrix as well as bigger, thicker and more interconnected hyphal forms. *C. parapsilosis* biofilms were composed solely by aggregates cell and filamentous forms which increased in size as the glucose concentration increased.

FKS1 gene expression increased for both *C. albicans* and *C. parapsilosis* strains as the glucose level in the medium increased, being more expressed, in general, in the first specie. Thus, these results support that *FKS1* gene expression is glucose dependent which suggest a high glucan content in the ECM of *C. albicans. BCR1* gene expression was not glucose dependent, being expressed similarly in the total absence of glucose for both *C. albicans* and *C. parapsilosis* strains. Suggesting that cell adhesion may be independent, to some level, of the glucose or glutamine present in the growth medium. However, its expression did increase for all strain with the increase in glucose, suggesting that an increase in the nutrient concentration can influence the amount of surface adhered cells.

Briefly, this work highlights the heterogeneous susceptibility of different *Candida* species and respective strains to glucose concentration variation and glutamine presence/absence in the growth medium, in terms of biofilm formation ability. It showed that, even when forming low amounts of biofilms, the number of adhered live cells to the surface remains unaltered for most conditions, proving that an increase in glucose from 0.2% to 10% does not affect cell viability negatively. The presence of glutamine is not vital to the growing cells in RPMI medium, but the absence of glucose can be, especially in the case of oral isolates. Further work is necessary to exactly understand the mechanisms behind biofilm formation, in different concentrations of glucose and the diverse nature of the structure of the formed biofilms, in order to better understand the mechanisms that allow to *Candida* cells to survive in such different conditions.

6 – Future perspectives

This chapter describes the further directions that can be taken in order to more completely understand the effect of glucose variation and glutamine presence in the growth medium

This work provided a clear perspective of the diverse nature of several *Candida* species, highlighting different susceptibilities and hitting at different metabolic processes and physiologies. A few suggestions that could be taken into consideration for further studies are:

a) Further exploration of biofilm forming ability by several *Candida* species, in mediums with higher concentrations of glutamine, comparatively to the biofilms formed in mediums with only an equivalent amount of glucose;

b) Assessment of biofilm ECM and cell wall composition of several *Candida* species in mediums with different glucose concentration and in the absence/presence of glutamine for a better understanding of the differences between the biofilms formed between the studied species, by a proteomic approach;

c) Assessment of antifungical resistance of biofilms of several *Candida* species formed in the studied conditions;

d) Evaluation of the effects of glucose variation in the browth medium as well as its substitution by glutamine in the Zap1 alternative pathway for glucan incorporation in biofilm matrix, by Real-time PCR determination of the gene;

e) Assessment of the effect glucose and glutamine in the expression of *FKS1*, *BCR1* and *ZAP1* genes, in mutant created strains of several Candida species;

f) Assess the effect of glucose in colonization and invasion of reconstituted human oral epithelium, , for several *Candida* species.

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Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth.

In A journey to the center of the earth

Julius Verne, 1864