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The impact of carbon source in *Candida albicans* virulence: participation of *RLM1* in pathogen host interaction

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Professora Doutora Paula Sampaio
e da
Professora Doutora Célia Pais

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DECLARAÇÃO

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Abstract

The survival of all microbes depends upon their ability to respond to environmental challenges. To establish infection, pathogens such as *Candida albicans* must support effective stress responses to counter host defenses while adapting to dynamic changes in nutrient status within host niches. Studies of *C. albicans* stress adaptation have generally been performed on glucose-grown cells, leaving the effects of alternative carbon sources upon stress resistance largely unexplored as well alterations in its virulence.

In a previous work both copies of the *RLM1* gene of *C. albicans* were deleted and phenotypic analysis of Δ*rlm1*/Δ*rlm1* mutants showed typical cell wall weakening phenotypes, such as hypersensitivity to Congo Red, Calcofluor White and caspofungin, showing its involvement in the cell wall remodelling. To understand the role of *RLM1* under the influence of different carbon source, phenotypic characterization against cell wall damaging stress agents, modulation of virulence factors as well as their involvement in host-pathogen interaction was preformed in the present work.

Candida albicans Δ*rlm1*/Δ*rlm1* mutant displayed phenotypes associated to cell wall deficiency such as, hypersensitivity to Congo red, caspofungin in glucose- and lactate- grown cells. However the *RLM1* mutants were slight more resistant to Congo Red when grown in lactate. On the other hand lactate-grown cells were not able to growth al all in the presence of SDS and presented sensitivity to caffeine, in comparison with glucose-grown cells. The increased transcription of genes already reported to be involved in cell adhesion correlated well with adhesion and biofilm assays, in which *RLM1* mutant presented greater biofilm formation than WT in both growth condition. However cell adapted to lactate adhered more and biofilm formation was more pronounced. The host-pathogen interaction was accessed by co-incubation with murine macrophages-like cell line (J774). In general, lactate-grown cells were less efficiently killed in comparison to glucose-grown cells. However the mutant presented distinct behaviors, they were more resistant when adapted to glucose than to lactate. The TNF-α and IL-10 were lower in response to *Δrlm1/Δrlm1* mutant and the cellular toxicity, measured as extracellular lactate dehydrogenase activity, was significantly lower in comparison with the WT and complemented strains in glucose-grown cells. The effect observed before was reverted when *C. albicans* cells were grown on lactate.

In conclusion, *C. albicans* cells adapted to different carbon sources behave differently, particularly in the interaction with macrophages, in which the RLM1 mutation plays a decisive effect since it affects the cell wall integrity.

Resumo

Os microrganismos para sobreviver dependem da capacidade de responder aos desafios apresentados pelo ambiente onde estão inseridos. O sucesso da infecção por parte dos agentes patogénicos, tais como *Candida albicans,* assenta na capacidade de responder eficazmente a stresses causados pelas defesas do hospedeiro e à adaptação a alterações constantes dos nutrientes no hospedeiro. Alguns estudos indicam que o crescimento em fontes de carbono alternativas tem influência na resistência ao stress e alteram a virulência de *C. albicans*.

Em trabalhos anteriores, o gene *RLM1* de *C. albicans* foi deletado e a análise fenotípica dos mutantes *Δrlm1/Δrlm1* revelou que estes apresentam hipersensibilidade ao *Congo Red*, *Calcofluor white* e Caspofungina, fenótipos típicos de fragilidade na parede celular. De forma a perceber o papel de *RLM1* em células adaptadas a diferentes fontes de carbono, foi feita uma caracterização fenotípica usando agentes que perturbam a parede celular, avaliada a modulação de alguns fatores de virulência, bem como a interação hospedeiro-patogéno.

Os mutantes *Δrlm1/Δrlm1* de *Candida albicans* crescidos em glucose e láctico exibiram fenótipos associados a deficiências na parede celular, tais como, hipersensibilidade ao *Congo Red* e Caspofungina. No entanto, estas revelaram ser ligeiramente mais resistentes ao *Congo Red* quando crescidas em láctico. Por outro lado, as células crescidas em ácido láctico apresentaram maior dificuldade em crescer na presença de cafeína, não tendo sido detectado crescimento em SDS, contrariamente às células crescidas em glucose que não apresentaram sensibilidade a estes stresses. O aumento da expressão de genes envolvidos na adesão célular correlacionou-se diretamente com os ensaios de adesão e biofilme, em que os mutantes *Δrlm1/Δrlm1* apresentam uma maior formação de biofilme em comparação com a estirpe selvagem em ambas condições de crescimento. Contudo, células crescidas em ácido láctico aderiram mais e a formação de biofilme foi mais evidente. A interação *Candida*-hospedeiro foi avaliada através da co-incubação com a linha celular de macrófagos J774. Em geral, as células crescidas em ácido láctico foram fagocitadas de forma menos eficiente em comparação com células crescidas em glucose. As estirpes mutante *Δrlm1/Δrlm1* apresentaram comportamentos distintos quando crescidas em diferentes fontes de carbono; em glucose os níveis de produção de TNF-α e IL-10 foram mais baixos e a toxicidade celular, avaliada pela atividade da lactato desidrogenase, foi significativamente mais baixa, em comparação com as estirpes selvagem e complementadas. O efeito anteriormente observado foi totalmente revertido quando o mutante foi crescido em meio com ácido láctico.

Em conclusão, células de *C. albicans* a diferentes fontes de carbono apresentam comportamento diferentes, nomeadamente na interação com macrófagos, onde o gene *RLM1* parece ter um papel decisivo pois afecta a integridade da parede celular

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Abbreviations and Acronyms list

A

APCs	Antigen Presenting Cells

B

BP	Base Pairs
BSA	Bovine Serum Albumin
BSIs	Bloodstream Infections

C

cDNA	Complementary Deoxyribonucleic Acid
CFG	Caspofungine
CFU	Colny Forming Unit
CFW	Calcofluor White
CLRs	C-Type Lectin Receptors
COX2	Cyclooxygenase 2
CR	Congo Red
CV	Crystal Violet
CWI	Cell Wall Integrity
CWP	Cell Wall Protein

D

DMEM	Dulbecco's Modified Eagle's Medium

E

ELISA	Enzyme Linked Immune Sorbent Assay

F

FBS	Fetal Bovine Serum
FCM	Flow Citometry
FNIs	Fungal Nosocomial Infections

FSC Forward Scatter

G

GDP Glyceraldehyde-3-Phosphate Dehydrogenase

GFP Green Fluorescent Protein

GI Gastrointestinal

GPI Glycosyl Phosphatidyl Inositol

H

HEPES 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

HIV Human Immunodeficiency Virus

HSCT Hematopoietic Stem Cell Transplants

I

IFN-γ Gamma Interferon

IgG Immunogloblin G

IL Interleucine

L

LDH Lactate Dehydrogenase

LIP Lipases

M

MADS Mcm1, Argamous-Deficiens-Serum Response Factor

MAPK Mitogen-Activated Protein Kinase

MHC Major Histocompatibility Complex

N

NLR Nucleotide Leucine-Rich

O

OD Optical Density

OPC Oropharyngeal Candidiasis

ORF Open Reading Frame

P

PAMPs Pathogen Associated Molecular Patterns

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PI Propidium iodide

PKC Cell Integrity Or Protein Kinase C

PLs Phospholipases

PRRs Pattern Recognition Receptors

Q

qRT-PCR Real-Time Reverse-Transcription Polymerase Chain Reaction

R

RNA Ribonucleic Acid

ROS Reactive Oxygen Species

S

SAP Secreted Aspartyl Proteinases

SBF Swi4-Swi6 Cell Cycle Box Binding Factor

SDS Sodium Dodecyl Sulfate

SEM Scanning Electron Microscope

SSC Side Scatter

T

TCR T-Cell Receptor

TGF-β Transforming Growth Factor Beta

Th T-Helper

TLRs Toll-Like Receptors

TNF-α Tumor Necrosis Factor A

U

USA	United States of America

W

WO	White Opaque
WT	Wild Type

Y

YCB	Yeast Carbon Base
YE	Yeast Extract
YNB	Bacto Yeast Nitrogen Base Without Amino Acids
YPD	Yeast Extract, Peptone And Dextrose

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Objective

1. Background

1.1. Epidemiology of invasive fungal infection

Over the last decades, invasive fungal infections, particularly fungal nosocomial infections (FNIs), have assumed a great importance. This phenomenon is essentially due to the increasing population at risk. People with human immunodeficiency virus (HIV) infection, recipients of solid organ or hematopoietic stem cell transplants (HSCT), patients with hematologic malignancies, burns or indwelling medical devices and low-birth weight infants are the most susceptible set of population to these kind of infections (Warnock 2007; Sabino et al. 2010). In the United States of America (USA), from 5231 cases of sepsis in 1979 to 16042 cases in 2000 (207% raze) were caused by fungal infections as reported in the retrospective study of Martin and colleagues (Martin et al., 2003). The advances of medicine, surgery and transplantology is thus one of the major causes of the dramatic increase in the number of immunocompromised individuals who are more susceptible to FNIs, including aspergillosis, candidiasis, cryptococcosis, and zygomycosis (Warnock 2007; Sabino et al. 2010). Currently, many of these patients die not because of their underlying illness but as a result of a deep-seated fungal infection (Tortorano et al. 2004).

Since these infections are among the main cause of morbidity and mortality, leading to an increase in the hospitalization time and, consequently, high costs associated to patients' treatment, the FNIs are indeed a serious public health problem (Lass-Florl 2009; Hota 2004; Gudlaugsson et al. 2003).

Fungal infections can either be superficial, affecting the skin, hair, nails and mucosal membranes, or systemic, involving major body organs (Ruping et al. 2008). Besides, these kind of infections have been reported as great threat in the intensive care units (ICUs), where the incidence is much superior than in the general population of hospitalized patients (Bassetti et al. 2006; Cheng et al. 2005; Vaz et al. 2011).

In addition to factors related to the hospital unit and underlying disease involving the patient, factors related to the microorganism are of major importance to the progression of hospital acquired infections. In specific situations, such as under immunosuppression, microorganisms of the normal microbiot can be responsible for most of the nosocomial infections (Hota 2004; Eggimann et al. 2003).

A significant cause of infection entails several species of *Candida*. More than 17 different species of *Candida* have been identified as etiologic agents of bloodstream infections (BSIs). However, approximately 95% of all *Candida* BSIs are caused by only four *Candida* species: *Candida*

albicans, *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis* (Hajjeh et al. 2004; Ostrosky-Zeichner et al. 2003; Pfaller et al. 2004). Although *C. albicans* remains the most important cause of invasive candidiasis, the non-*albicans Candida* (NAC) species are gaining clinical importance once they constitute about 50% of the reported cases of candidemia (Krcmery and Barnes 2002).

In Europe, fungal pathogens represent the major eukaryotic agents of serious infection, in which *Candida albicans* and other emergent *Candida* species have become the third most frequent cause of nosocomial blood stream infections (Perlroth et al. 2007; Pannanusorn et al. 2013). These yeasts may reside in the host as benign commensals however, they may take advantage of a locally or systemically debilitated immune system to proliferate and cause disease. Moreover, new extracorporeal reservoirs colonized by *Candida* species have been identified in recent years, as natural environments, inanimate surfaces, health professionals' hands and hospital's surface. This fact supports and clarifies some recent evidences of the epidemiological cycle of hospital infections caused by *Candida* species of exogenous origin (Vaz et al. 2011). In fact, some studies have showed that the hands of health professionals may be colonized by yeasts and also serve as reservoirs of nosocomial infections (NIs) (Vaz et al. 2011; Ferreira et al. 2013; Sabino et al. 2010). The use of catheters and other medical devices is also related to development of nosocomial infections (NIs) with *Candida* species via exogenous routes, because the high prevalence of *Candida* isolates in samples from catheter tips may have an important role in the spread, progression and persistence of NIs (Ferreira et al. 2013; Traore et al. 2002; Passos et al. 2005). However, the *Candida* species are not related exclusively to hospital candidemia, because hematogenous infections also occur in the community (Ferreira et al. 2013).

In Portugal there are few studies regarding the epidemiology of candidiasis, but in 2010, a work developed by Sabino et al (Sabino et al. 2010) studied the incidence of candidemia in a Portuguese oncology hospital during 6 years. In this study, it was demonstrated that the most frequently species found were *C. albicans* followed by *C. parapsilosis*, *C. tropicalis*, *C. krusei* and *C. glabrata.* Furthermore, it was assessed that the mortality rate associated of candidemia was 31,9% (Sabino et al. 2010). Given their increasing frequency and high morbidity and mortality rates, prevention of invasive fungal infections has become of paramount importance. Therefore, host-fungus interaction studies may contribute to understand the epidemiology and pathogenesis of these infections and help develop new strategies to fight fungal infections.

1.1.1. *Candida albicans*, the major model yeast of pathogenicity

There are known approximately 200 species known in the genus *Candida* but only a few species have been identified as pathogenic. Macroscopically, colonies of *Candida* spp. are cream-colored to yellowish. All species produces blastoconidia and many of them are dimorphic growing as budding yeast cells, pseudohyphae or true hyphae (Eggimann et al. 2003).

Since the dawn of civilization the growth of the opportunistic fungal pathogen *Candida albicans* on the surface of human tissue was noted and described by Hippocrates in the fourth century B.C. (Kumamoto and Vinces 2005).

Candida albicans is an ubiquitous and dimorphic fungus that exists as a commensal in warm-blooded animals, including humans. It colonizes mucosal surfaces of the oral and vaginal cavities and the digestive tract. However, if the balance of the normal flora is disrupted or the immune defenses are compromised, *C. albicans* can switch from commensal to pathogenic, being capable of infecting a variety of tissues and causing fatal systemic diseases (Sampaio et al. 2010).

The diploid *C. albicans* has eight pairs of homologous chromosomes, with a genome size of 14.3 Mb, based on genome sequencing of SC5314 strain. The content of GC in *C. albicans* genome is about 33.5%, 6438 genes, an average gene size of 1,468 bp and an intergenic average size of 858 bp (Magee 1993; Butler et al. 2009). Another distinctive feature of *C. albicans* is an alteration in its genetic code in which the codon CUG encodes serine rather than leucine (Santos and Tuite 1995). It shares this property with a group of related species that are collectively known as the CUG clade (Butler et al. 2009). This clade includes other pathogenic yeast such as *C. paropsilosis* and *C. tropicalis*. This characteristic increases dramatically the number of different proteins encoded by the 6438 *C. albicans* genes and results in an extensive phenotypic variability considered as among others, an attribute of virulence (Gomes et al. 2007).

1.1.1.1 Virulence factors of C. albicans

Virulence can be defined as the capacity of some microorganism cause disease, and is highly regulated by many factors. In order to establish a variety of infections, pathogens have to evade the immune system, survive, divide in the host environment and spread to new tissues (Yang 2003). Thus, virulence describes the degree of pathogenicity, where some strains can be more or less virulent than the others. *C. albicans* can survive in several anatomically distinct sites and the putative success of conversion from an innocuous commensal organism to an opportunistic pathogen depends on several virulence factors (Jayatilake 2011; Calderone 2004; Gow et al.

2012). These attribute of virulence include adherence to host cells, morphogenesis, biofilm formation, phenotypic switching and secretion of degradative enzymes (Calderone and Fonzi 2001; Schaller et al. 2005; Chauhan et al. 2006; Lim et al. 2012).

1.1.1.1.1 Adhesion

The adhesion ability of *Candida* cells to the host cells or to medical-devices is an important trait to begin the cycle from colonization to the establishment of the infection. The level of adhesion is dependent on host, microbial and abiotic surface proprieties, such as cell wall composition or cell-surface hydrophobicity (Silva et al. 2011).

In *Candida* species, adhesion is mediated by some well characterized adhesion factors such as the proteins Hwp1, Eap1, all members of the Als family, and others (Calderone and Fonzi 2001). Several of these are glycosylphosphatidylinositol (GPI)-proteins (linked to cell wall glucan) or GPI-anchored proteins (linked to the cell membrane), which are exposed at the cell surface (Gow and Hube 2012b). Mutants lacking these adhesion genes display decreased adherence to host substrates in vitro as well as a corresponding virulence reduction in several in vivo experimental models of candidiasis (Sheppard et al. 2004).

As previously referred, one of the most well-studied adhesins are the agglutinin-like sequence (Als) proteins family, encoded by eight *ALS* genes (*ALS*1-7 and *ALS* 9) (Yang 2003). Some differences in N-terminal domain among distinct Als proteins leads to differences in their function. In early stages of infection, Als1p has been reported to mediate binding to human vascular endothelial cells and epithelial cells, while Als5p is responsible for collagen, fibronectin, bovine serum albumin and laminin adhesion (Sheppard et al. 2004).

C. albicans can express all eight ALS genes, but *C. parapsilosis* and *C. tropicalis* express only five and three ALS genes, respectively (Silva et al. 2011).

1.1.1.1.2. Morphogenesis

Another striking feature of *C. albicans* is its ability to grow as an unicellular budding yeast (blastopores) and as a filamentous form (hyphae or pseudohyphae) (Sudbery 2011). This ability, represented in Figure 1, is tightly regulated by signal transduction pathways in response to environmental stimuli such as pH, temperature, or different compounds such as N-acetylglucosamine or proline. Perhaps the most critical criterion for pathogenicity is the induction of the mycelial form by serum and during the interaction with macrophage cells (Jacobsen et al. 2012).

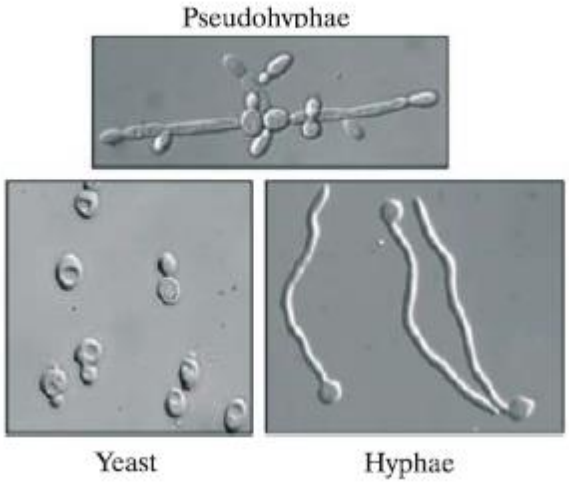

Figure 1. Distinctive morphology of *C. albicans*. Representation of the three different morphologies that *C. albicans* can acquired. Differential interference contrast (DIC) images of cells with hyphae, pseudohyphae, and yeast morphologies adapted from (Kim and Sudbery 2011).

Some studies suggest that budding yeast cells are suitable for dissemination while hyphae are essential for adaptation to different host environment conditions and also for tissue and organ invasion (Mavor et al. 2005; Lim et al. 2012).

The ability of hyphae formation is observed in some species of *Candida* such as *C. albicans*, *C. parapsilosis* or *C. tropicalis* and is considered to be crucial for evasion of host defenses (Lim et al. 2012). *C. glabrata* is commonly known and described as unable to form hyphae and pseudohyphae but still is able to induce infection, indication that other virulence factors may take the lead (Odds et al. 1997; Lachke et al. 2002; Csank and Haynes 2000).

1.1.1.1.3. Extracellular hydrolytic enzymes

C. albicans as well as other *Candida* species can secret hydrolytic enzymes. This feature is considered a virulence factor and required during *Candida* infections. Secreted aspartyl proteinases (Sap), phospholipases and lipases are the most important extracellular enzymes secreted by *Candida* species (Mavor et al. 2005; Jayatilake 2011). These enzymes are needed to degrade host cell surface molecules to invade host cells by digesting its membranes, to resist to host immunity by attacking immune system cells and molecules, such as heavy chains of IgG, C3 protein, keratin, collagen, albumin, fibronectin, hemoglobin among others, and for nutrient acquisition (Yang 2003; Trofa et al. 2008).

The secreted aspartyl proteinases family is composed by 10 *SAP* genes that encode the Sap proteins. Some studies carried out by Schaller and co-workers (Schaller et al. 2001) demonstrated that SAPs are differentially expressed in yeast and hyphal form and in phenotypically switched states. For example, SAP1-3 is mainly expressed on cell wall and cytoplasm of blastopores, SAP4-6 is restricted at the tips of hyphae and phenotypically switched cells express SAP1 and SAP3. Furthermore, SAP8 is mostly detected in yeast cells grown at 25°C and SAP9 is preferentially expressed in later growth phases (Yang 2003). In this sense, the flexibility of *SAP* genes expression may be contribute to the success of *Candida* species as an opportunistic pathogen, by allowing the fungus to survive and to cause infections on different host tissues and different growth conditions (Naglik et al. 2003).

Other hydrolytic enzymes responsible for conferring virulence to *Candida* species are phospholipases (PLs). These enzymes hydrolyze phospholipids to fatty acids and glycerol. The classification of these enzymes is based on the different ester bonds cleaved being classified as PLs A, B, C and D. However, it has been demonstrated that only proteins encoded by the phospholipase B family (*PLB1-5)* seem to be extracellular, particularly *PLB1* that plays a key role in virulence in animal models of candidiasis (Calderone and Fonzi 2001; Silva et al. 2011). The expression of this enzymes family during infection could lead to host cell membrane impairment and *Candida* cells adhesion.

Several studies demonstrated that PLs are differentially expressed. For example the PLs can be expressed at the tips of *Candida* hyphae and on the initial buds of *C. albicans* cells during invasion (Samaranayake et al. 2005; Jayatilake et al. 2005; Ghannoum 2000). These studies confirm PLs involvement in pathogenesis by enhancing tissue penetration. Other studies showed

that *C. tropicalis* and *C. parapsilosis* are also capable of producing extracellular PLs, although in much lower levels than *C. albicans*. PL activity was not so clear for *C. glabrata* and *C. krusei.*

Lipases are involved in the hydrolysis and synthesis of triacylglycerols. Ten *LIP* genes (*LIP1-10*) encode these enzymes, and studies confirmed that they are differentially expressed at different stages and sites of infection (Trofa et al. 2008). Only in *C. albicans* and *C. tropicalis* all ten *LIP* genes (*LIP1-10*) were detected. For *C. parapsilosis*, only two lipase genes, *LIP1-2*, have been reported (Trofa et al. 2008) and no studies regarding *LIP* genes expression in *C. glabrata* and *C. krusei* are available. Gácser and co-workers demonstrated that lipases are important for *Candida* virulence, by showing that with lipase inhibitors the tissue damage observed was significantly reduced during infection in reconstituted human tissue models (Gacser et al. 2007).

1.1.1.1.4. Phenotype switching

Candida species can undergo a phenomenon called phenotypic switching (Slutsky et al. 1985). There are a number of different classes of switching; one of the most well-characterized is white-opaque switching (Slutsky et al. 1987). The most common forms of *C. albicans* colonies are smooth, white, and dome-shaped, these colonies are called “white”. When a suspension of white phase cells are plated out, about 1 in 1,000 colonies will have a different appearance being flatten and having a grey color. Within these new colonies, cells are larger and have an oblong rather than opaque appearance. Scanning electron micrographs show that the surface of these cells is covered in pimples. These cells are said to be in the “opaque” phase. Opaque cells will switch back to white again with a frequency of about 1:1,000 and this switching process is believed to be under epigenetic control (Kim and Sudbery 2011).

Strain WO-1 of *C. albicans* is the most studied phenotypic switching to white-opaque phases. Studies involving ultra-structural observations of WO-1 white-opaque phenotypes have showed differences in cell shape, cell surface structures and growth at 37°C, suggesting that phenotypic switching could affect the virulence behavior of the organisms (Kim and Sudbery 2011).

This ability is also observed in *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. Laffey and co-workers (Laffey and Butler 2005) identified four main phenotypes in *C. parapsilosis* colonies, including the concentric, crepe, smooth and crater phases and confirmed their relation with biofilms formation. Also, they identified four phenotypes in *C. glabrata* (white, dark brown, very dark brown and light brown) and França and co-workers demonstrated the presence of also four

possible phenotypes in *C. tropicalis* (smooth, rough, ring, semi-smooth) (Franca et al. 2011; Lachke et al. 2002). Phenotypic switching in *C. krusei* has not been reported yet.

1.1.1.1.6. Biofilm formation

Biofilms are surface-associated communities of cells surrounded by an extracellular matrix and constitute an increasing problem in the clinical setting (Blankenship and Mitchell 2006). *C. albicans* can form biofilms on natural surfaces, such as teeth, and on foreign surfaces, such as implanted devices. Due to the resistance of these biofilms to the common antimicrobial therapy, treatment often requires removal of infected devices to avoid potentially fatal consequences (Nobile and Mitchell 2006; Blankenship and Mitchell 2006; Kojic and Darouiche 2004; Douglas 2003).

In an initial stage, yeast cells adhere to the solid surface. Then, they undergo morphogenesis to produce a dense layer of cells of mixed morphology embedded in an extracellular matrix rich in secreted cell wall polymer ß-1,3 glucan. *In vivo*, it was shown that the biofilm can protects cells against host defenses (Nobile et al. 2009).

Some of the genes involved in biofilm formation were identified, as being also required for hyphal formation, such as the transcription factor Efg1 and the kinase Yak1. Others are apparently specific to biofilm formation, the zinc finger transcription factor Zap1, that is thought to regulate the production of matrix polysaccharide components and the transcription factor Bcr1, that regulates the formation of adhesens that allow yeast cells to stick to the solid surface (Nobile et al. 2009).

During disease establishment and progression, these virulence factors are assumed to be required in differing extents (Brown and Gow 1999; Huang 2012). Overall, the interaction between *C. albicans* virulence factors and host defense mechanisms plays a crucial role in determining whether colonization remains harmless or leads to infection of the epithelium and ultimately to systemic (Calderone and Fonzi 2001; Chauhan et al. 2006; Lim et al. 2012; Schaller et al. 2005).

1.1.2. Host response to fungal infections

The human host immune system is sophisticated and efficient being responsible for controlling and eliminating infectious microorganisms. The first line of defense for pathogenic agents like *C. albicans* is formed immediately by the innate immune system. However, the intervention of the adaptive immune system is required.

1.1.2.1. The role of innate immune

The host innate immune system is responsible for a constant and repetitive response that is specific, highly complex, and usually also highly efficient. After pathogen recognition, the immune system is activated and generates effector functions in order to eliminate the infectious microbe, which normally is well succeeded. Providing the first line of immune defense, the innate immune system is both central and critical for the complete host immune response. The innate immune defense affords 3 major effector mechanisms, (1) the complement system, (2) antimicrobial peptides, and (3) immune cells that recognize and respond to foreign microbes. Therefore, in order to survive or invade an immunocompetent host, any pathogen needs to cross the multiple borders of innate immunity (Zipfel et al. 2007).

The cellular mechanism will be further detailed in this thesis.

1.1.2.1.1. A cellular-dependent response: Macrophages and Dendritic cells

Monocytes, macrophages and dendritic cells are the cell types most commonly related with the innate immune response against *C. albicans* infection. Phagocytic cells have multiple receptors that recognize *C. albicans* cells.

Phagocytosis is induced and regulated by the pattern recognition receptors (PRRs) on the surface of antigen presenting cells (APCs) that recognize pathogen associated molecular patterns (PAMPs) (Hoebe et al. 2004; Akira and Takeda 2004; Akira et al. 2001). The recognition of PAMPs leads to microbial uptake and, consecutively, to a modulation of gene expression to initiate an adaptive and target-oriented immune response (Gauglitz et al. 2012). So far, three major groups of PRRs have been distinguished: toll-like receptors (TLRs), C-type lectin receptors (CLRs) and nucleotide binding domain, leucine-rich repeat containing (NLR) protein family as shown in Figure 2 (Gauglitz et al. 2012).

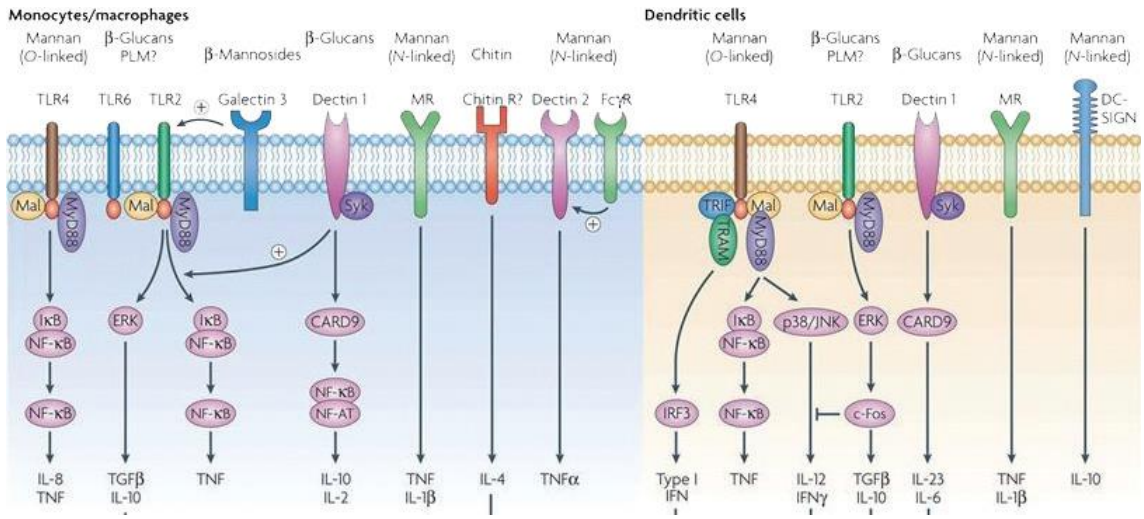

Figure 2. The major pattern-recognition receptors (PRRs) involved in the recognition of specific *C. albicans* pathogen-associated molecular patterns (PAMPs). Stimulation of host response by *C. albicans* at the cell membrane is mediated by toll-like receptors (TLRs) and C-type lectin receptors (CLRs) adapted from (Jouault et al. 2003).

The mannose receptor and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin homolog (SIGN)-related 1 (SIGNR1) are two examples of these receptors (Cambi et al. 2003; Marodi et al. 1991; Filler 2006). Dectin-1 is another known receptor for *C. albicans* (Brown et al. 2003). This receptor can be found on macrophages and dendritic cells, and recognizes β-glucans, a component of the *C. albicans* cell wall. Even though, not being specific for macrophages binding to blastospores, this receptor is required for an optimal phagocytosis of *C. albicans* blastospores. Remarkably, when *C. albicans* cells produce hyphae, they do not express β-glucan on their surface and so binding to dectin-1 is limited. This evidence suggests that the β-glucans masking on the surface of hyphae may help preventing recognition (Filler 2006).

Incubating macrophages with *C. albicans* blastospores, results in the blastospores phagocytosis and secretion of pro-inflammatory mediators. *C. albicans* blastospores binding to dectin-1 on macrophages results in the secretion of TNF-α and arachidonic acid, and in increased expression of COX2 (cyclooxygenase 2) (Brown et al. 2003; Suram et al. 2006), which result in a pro-inflammatory response. Interestingly, phagocyte response to *C. albicans* hyphae may be different than to blastospores and a complete anti-inflammatory outcome may be induced (Figure 3). Moreover, members within one family of PRRs can trigger opposite signaling effects, indicating that the ultimate outcome of a pathogen-induced immune response depends on the pathogen signature and the PRRs involved (Gauglitz et al. 2012).

As depicted in Figure 3, each ligand–receptor system activates specific intracellular signaling pathways that lead to modulation of several components of the host immune response (Jouault et al. 2003).

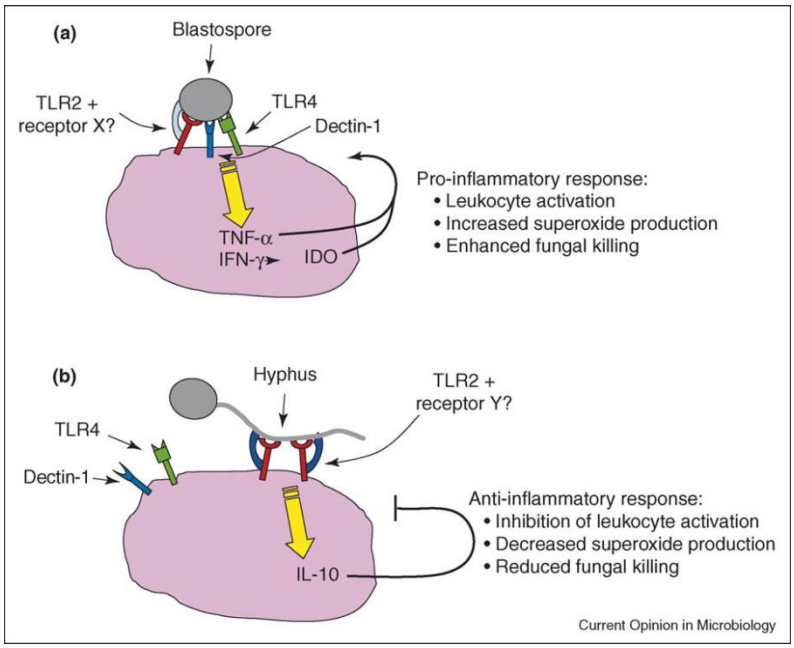

Figure 3. Recognition of *C. albicans* blastospores and hyphae by monocytes and macrophages receptors.

Reactive oxygen species (ROS) are produced by macrophages in response to blastospores phagocytosis in order to kill pathogens. However, under most in vitro experimental conditions, human macrophages have limited ability to kill *C. albicans*, even though they avidly phagocyte the microorganism. Newman et al. (Newman et al. 2005) discovered that one reason for this incomplete killing ability is that living *C. albicans* blastospores inhibit the phagolysosomal fusion in macrophages. The exact mechanism for this inhibition has not yet been determined. Interestingly, if macrophages are adherent to a collagen matrix the phagolysosomal fusion is not inhibited, suggesting that the exposed extracellular matrix proteins have an import role on *C. albicans* immune evasive strategies (Newman et al. 2005).

1.1.2.2. The role of adaptive immune system

Additionally, fungal cells induce the adaptive immune response which involves antigen-specific T and B cells (Zipfel et al. 2011).

Three signals are crucial for effective stimulation of an adaptive immune response: (1) the presentation of antigens by APCs on the context of MHC molecules/T-cell receptor (TCR) on CD4+

T-cell, (2) the up-regulation of co-stimulatory molecules, and (3) production of cytokines that determine differentiation of a certain type of helper T-cell (Th) response. Also, it is important to refer that the complete integrated response to a specific pathogen depends on the mosaic of PRRs and receptor complexes that are involved (Jouault et al. 2003; Netea and Marodi 2010). Co-stimulation via multiple PAMPs–PRRs interactions may increase sensitivity and specificity of the immune recognition process as well as the type of adaptive immune response, as demonstrated in Figure 4 (Jouault et al. 2003).

PRRs activation induces production of a cytokine environment that will induce an adaptive immune response directed towards a Th1, Th2 or Th17 phenotype (Kapsenberg 2003; Napolitani et al. 2005; Kubin et al. 1994).

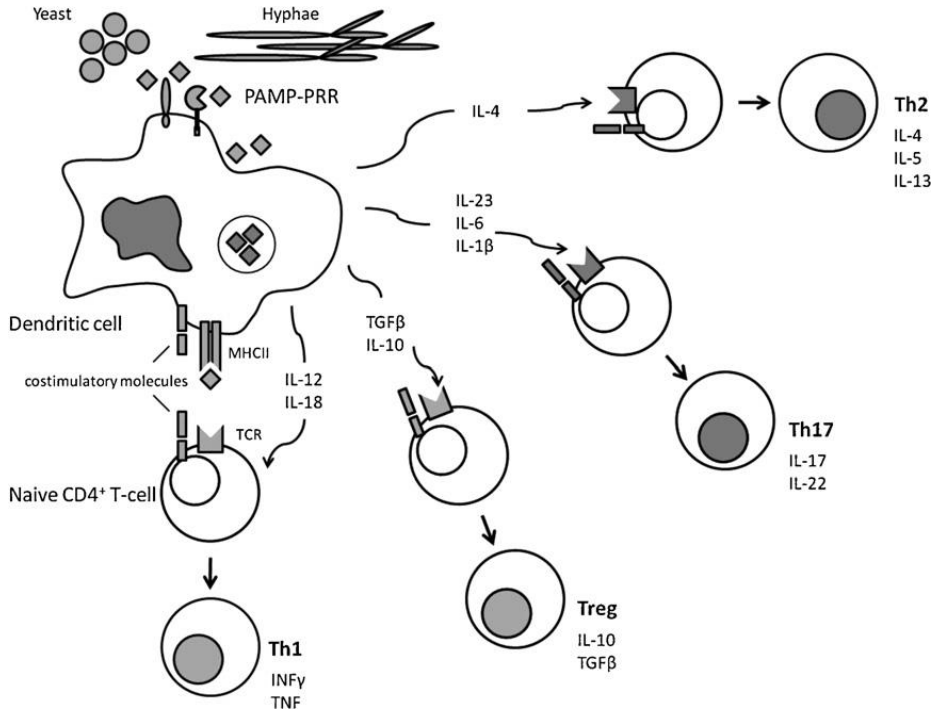

Figure 4. Modulation of T-cell responses by innate immune system. Engagement of pathogen associated molecular patterns (PAMPs) from *Candida albicans* by pattern recognition receptors (PRRs) lead to the induction of specific cytokine profiles regulating the differentiation of naïve CD4+ T-cells in to Th1 (T-helper 1), Th2 (T-helper 2), Th17 (IL-17 secreting T-helper) or Treg (T-regulatory) cells. For activating naïve CD4+ T-cells, presentation of antigens on MHC class II to T-cell receptor (TCR) is necessary in combination.

1.1.2.2.1. Th1, Th2 a central paradox and the identification of a new subset of T cells: Th17

After cell engulfment fungal antigens are processed by dendritic cells and transported to lymph nodes and presented to T helper (Th) and regulatory T cells (Brown 2011) that ultimately will induce the correct adaptive response.

In 1995, studies in mucosal candidiasis using the mouse model of gastric *Candida* infection showed that both, IFN-γ and IL-5 producing CD4+ T cells found in Peyer's Patches and mesenteric lymph nodes, were correlated to Th1 and Th2, respectively. Additionally, neutralization of IL-4 resulted in amplified IFN-γ production and boosted yeast clearance (Cenci et al. 1995; Hernandez-Santos and Gaffen 2012).

Other studies in oropharyngeal candidiasis (OPC) mouse model demonstrated that T cell-deficient mice were susceptible to infection, but could be protected by transferring CD4+ T cells (Farah et al. 2002). Cytokines related to Th1 cells, including IFN-γ and TNF-α, but not Th2 cytokines in the oral tissue, consistent with a Th1-based response. These results indicated that Th1 cells confer protective antifungal immunity, even though hallmark cytokines of both lineages, Th1 and Th2, were produced during infection (Hernandez-Santos and Gaffen 2012; Cenci et al. 1995; Cenci et al. 1998). However, depletion of CD8 or CD4 T cells in a susceptible mouse strain did not intensify oral colonization with *Candida* in the oropharyngeal candidiasis (OPC) mouse model (Ashman et al. 2003; Hernandez-Santos and Gaffen 2012), Therefore, T cells were involved in immunity to OPC, even though the specific subset and cytokines were still not well elucidated (Hernandez-Santos and Gaffen 2012).

In 1986 the Th1/Th2 paradigm was first described, and immune responses, whether infectious or autoimmune, were considered into these categories for approximately two decades. Over time, it became clear that this model was fraught with discrepancies.

A central paradox was that the signature Th1 effector cytokine, IFN-γ, was significantly less important in various disease settings than was IL-12, the key Th1 inductive cytokine (Steinman 2007). A significant renovation in the prevailing paradigm of CD4-mediated immunity occurred with the identification of Th17 cells, which settled many of these discrepancies. Subsequent studies showed that Th17 cells arise from inductive signals from TGF-β, IL-6, and IL-1b, while IL-23 is an essential maintenance and pathogenic factor for Th17 cells (Stockinger and Veldhoen 2007; McGeachy and Cua 2007).

Moreover, understanding the facts that regulate whether Th-response adopts a predominantly a Th1, Th2 or a Th17 type is crucial to prevent fungal infections (Chaplin 2006).

Knowing how pathogen and its host interact with each other is a crucial step for future fungal infections therapies.

1.1.3. Cell wall, first barrier or interface for host immune cells

The frontline of the pathogen–host interaction or contact is the fungal cell wall. Cell walls of fungal pathogens have an enormous importance in several biological processes. Being a dynamic structure, fungal cell wall is of great importance once (1) it protects from hostile environments, (2) it confers physical rigidity to maintain cell shape but also control morphogenesis during different stages of the fungus development, (3) it is responsible for the host immune recognition and for immune-avoidance, and (4) it maintains cellular integrity essential for cell growth (Kapteyn et al. 2000; Gow and Hube 2012a).

The fungal cell wall composition differs from each fungal species. In *C. albicans* example, two major layers constitute its cell wall, a skeletal inner layer (glucan and chitin) and a fibrillar outer layer (mannoproteins) (Bowman and Free 2006; Klis et al. 2001). The percentage of each *C. albicans* cell wall component is around 1–5 % chitin, 60–65 % glucan, and 35–40 % mannoproteins (per dry wall weight) (Klis et al. 2001; Munro et al. 1998) (Figure 5).

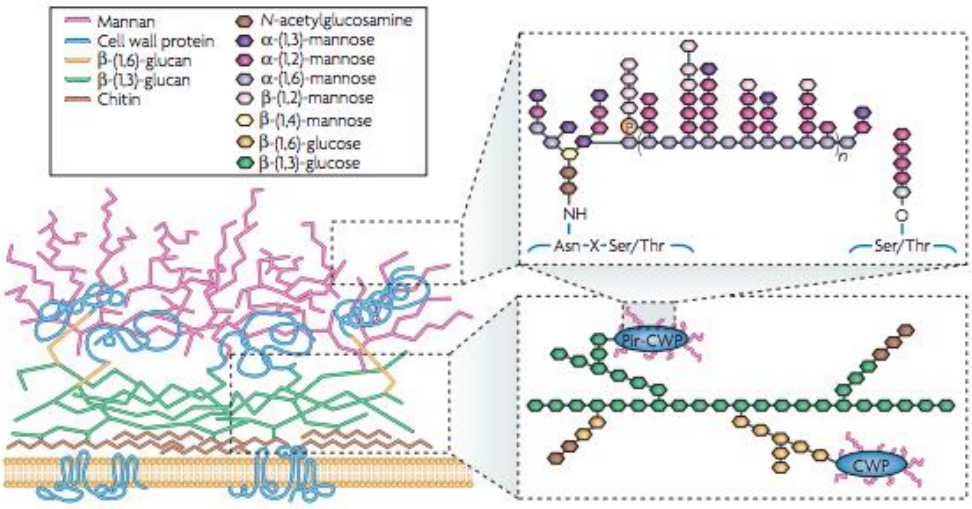

Figure 5. The structure of the *Candida albicans* cell wall. The schematic shows the major components of the cell wall and their distributions. β-(1,3)-glucan and chitin (poly-β-(1,4)-N-acetylglucosamine) are the main structural components, and these are located towards the inside of the cell wall. The outer layer is enriched with cell wall proteins (CWP) that are attached to this skeleton mainly via glycosylphosphatidylinositol remnants to β-(1,6)-glucan or, for mannoproteins with internal repeat domains (Pir-CWP), via alkali-sensitive linkages to β-(1,3)-glucan. The insets show the structure of the glucan and mannan components. Adapted from (Netea et al. 2008)

Hexose sugars such as glucose, mannose and galactose are used by *C. albicans* as preferential carbon source to produce energy and to synthesize the main components of cell wall the polysaccharides glucans, mannan and chitin (Lee and Munro 2014).

About a third of the total cellular biomass is constituted by cell wall, but fungal cells have to extremely coordinate the production of new cell wall material to allow cell growth. Several studies have demonstrated the influence of niche-specific metabolic regulation on cell wall remodeling in *C. albicans*, which implicates alterations in host-pathogen interactions and significant sensitivity to antifungal agents (Ene and Brown 2014; Ene et al. 2012a; Brock 2009).

According to Walker and colleagues, during *Candida* systemic infections in a rabbit model, transcriptional profiling shown alterations in the expression of genes associated with alternative carbon source metabolism, glucose assimilation, sugar transporters and cell wall surface remodeling (Walker et al. 2009). Genes associated with gluconeogenesis and the glyoxylate cycles are up-regulated when *C. albicans* and *C. glabrata* are exposed to human blood or neutrophils (Hube 2006; Fukuda et al. 2013; Lee and Munro 2014). Other studies suggest that starvation is relevant in vivo, for example, fluorescent reporter constructions have shown that the metabolic status of individual *C. albicans* cells within kidney lesions from a murine infection model can differ with some cells undergoing gluconeogenesis and others glycolysis (Barelle et al. 2006).

But still, the cell wall composition can be adaptably changed under stress conditions such as in response to certain environments or even when exposure to anti-fungal drugs. Several genes that involved in cell wall biosynthesis, remodeling and regulation have been shown to be vital for cell growth viability and pathogenicity (Lee and Munro 2014; Delgado-Silva et al. 2014).

1.1.4. The *RLM1* gene importance

The cell wall is a dynamic structure since it changes with alterations of the surrounding growth conditions and is remodeled as the cell increases in size and during morphogenetic processes such as mating, sporulation, or pseudohyphal growth. Upon cell wall damage, cells activate the cell wall integrity (CWI) mitogen-activated protein (MAP) kinase pathway (also known as the PKC pathway) so that the cell wall is repaired and cell integrity maintained. This response involves several processes (Figure 6).

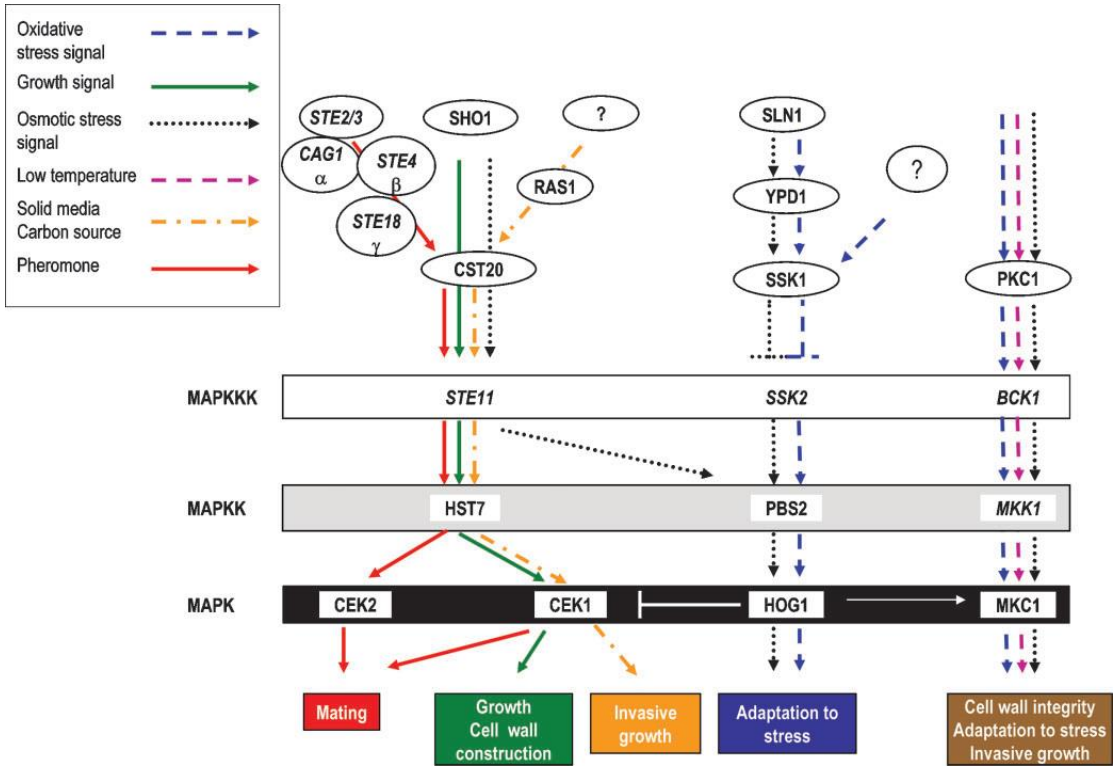

Figure 6. Main elements of the MAPK signal transduction network in *C. albicans*. Genes are represented in normal characters where deletion and/or phenotypic characterization of the corresponding strain has been carried out in C. albicans or, alternatively, in italic, where their position and role are just presumed based on the *S. cerevisiae* model and/or inspection of the genome. The existence of additional putative elements that may play a role in the activation of the corresponding MAPK is shown as question marks. The stimuli leading to the activation/repression of the pathways are shown either as stimulating (R) or inhibitory (–|) arrows. It is important to note that the arrows do not establish whether the interaction/crosstalk takes place through a direct or an indirect mechanism. The role of each MAPK in the physiological response is highlighted, but it must be taken into account that the interaction between pathways extends their indicated roles and generates a proper balanced response in the fungal cell. Adapted from (Monge et al. 2006)

The targets of the CWI pathway activation are the transcription factor heterodimer complex SBF (composed by Swi4 and Swi6), and the MADS-box transcription factor *RLM1* that activates genes involved in cell wall reinforcement and remodeling in response to cell wall stress (Lipke and

Ovalle 1998). Sampaio et. al showed that *C. albicans RLM1* presents a high allelic diversity, with many variants of the encoded proteins found in surveys of clinical isolates and that isolates with different *RLM1* allelic combinations presented different phenotypic characteristics (Sampaio et al. 2009). These studies indicated that *RLM1* is required for in vitro viability of *C. albicans* under caspofungin, and that inactivating this gene renders a described virulent strain completely avirulent (Delgado-Silva et al. 2014). Furthermore, an increase of cell adhesion binding proteins involved in biofilm formation was observed in *C. albicans* cells lacking *RLM1* gene (Delgado-Silva et al. 2014). The higher expression of adhesion proteins in the mutant strain raised a particular interest on the protein profile of *C. albicans* wild-type strain in comparison with the mutant that lacks this *RLM1* gene (Delgado-Silva et al. 2014).

1.2. Metabolism as a platform for *Candida albicans* pathogenicity

A few years ago, Brown and Gow suggested that a mechanistically link between the regulation of metabolism and virulence in *C. albicans* could exist (Brown and Gow 1999; Brown et al. 2000). It was thought that in response to the new microenvironments it encounters during disease establishment and progression, these links might allow this pathogen to adjust its metabolism in parallel with its set of virulence factors (Nowrousian 2014).

A fundamental aspect of infection is the ability of the pathogenic microorganism to assimilate and utilize essential nutrients from the host-niches. For instance, *C. albicans* that normally thrives as a relatively innocuous commensal organism within diverse niches like the skin, the oral cavity, and the gastrointestinal (GI) and urogenital tracts of most healthy individuals, must have a metabolic flexibility to assimilate and utilize the available nutrients in these distinctive niches (Nowrousian 2014; Brown et al. 2014a; Odds 1988; Calderone and Clancy 2012).

The study of *C. albicans* metabolism was initiated over fifty years ago (Van Neil and Cohen 1942) and in the 60's, enzymes of central carbon metabolism were characterized by Rao et al. (Rao et al. 1960).

Either directly or indirectly, studies about *C. albicans* metabolism have focused on virulence attributes or this in the mechanisms of action of antifungal drugs (Odds 1988). A good example is the attention that has been paid to the pathways involving ergosterol biosynthesis and cell wall biogenesis, both of them are antifungal targets (Sanglard et al. 2003; Shapiro et al. 2011).

Central and amino acid metabolism have been agreed as potential antifungal targets by many gene deletion studies in *C. albicans* (Roemer et al. 2003) and by transcript profiling studies (Lorenz

and Fink 2002, 2001). The main goal of the antifungal therapy is to kill the pathogen fungus rather than making it avirulent. Thus, metabolic process involving growth and survival in the host-niches represent a putative antifungal target and its study is of highly importance (Brock 2009). However, the validity of such targets and efficacy of potential drugs that hit these targets will depend on the extent of cross-reactivity with the host. Pathogenic microbes as *C. albicans* display a great flexibility in its metabolic program, which will enable it to adapt to different host microenvironments with different nutritional availabilities. So, an increase understanding about this field could provide crucial information that should facilitate the improvement of existing therapies and possibly the development of new therapeutic approaches.

Over the last years, significant efforts have been made to understand the metabolic requirements of *C. albicans* during infection. However, additional studies are crucial to appreciate the wide-ranging metabolic toolbox that contributes to the fitness of this pathogen and the extent to which metabolic adaptation is coordinated with the regulation of virulence attributes (Calderone and Clancy 2012).

Up until know, *Saccharomyces cerevisiae* has provided a reasonable metabolic paradigm for *C. albicans*. Even though, many metabolic pathways are conserved between *S. cerevisiae* and *C. albicans*, these organisms display significant differences in their metabolic programmes, which the most obvious is related to their patterns of sugar utilization (Askew et al. 2009; Nowrousian 2014; Brown et al. 2009). It is possible that the higher complexity and plasticity in the regulatory mechanisms that control metabolic processes reflects the evolutionary adaption of yeast to their respective niches and, in the case of *C. albicans*, to the diverse microenvironments it faces in the host during colonization and disease progression (Nowrousian 2014). These microenvironments are characterized as being complex, dynamic and frequently glucose-limited. The glucose levels are maintained at around 0.06–0.1% (3–5 mM) in the bloodstream, and are around 0.5% in vaginal secretions (Barelle et al. 2006; Owen and Katz 1999). *C. albicans* induces glycolytic, tricarboxylic acid cycle, and fatty acid β-oxidation genes during mucosal invasion (Zakikhany et al. 2007; Wilson et al. 2009). During renal infection and in the bloodstream, *C. albicans* populations are heterogeneous, and so, individual cells displays glycolytic activity (hexose catabolism) or gluconeogenic activity (hexose anabolism), depending upon their nearby microenvironments (Barelle et al. 2006; Fradin et al. 2003; Fradin et al. 2005). After phagocytosis by macrophages and neutrophils, *C. albicans* cells exhibit expression patterns that suggest carbon starvation, activating enzymes involved in fatty acid β-oxidation, the glyoxylate cycle, and gluconeogenesis

(Fradin et al. 2005; Lorenz et al. 2004; Rubin-Bejerano et al. 2003). In the GI colonization lactic acid metabolism is essential and this non-fermentable carboxylic acid is present at significant concentrations in the vagina (≈0.4%: 45 mM) (Ueno et al. 2011; Owen and Katz 1999). Hence, *C. albicans* thrive in host-niches that contain contrasting carbon sources and this metabolic adaptation is controlled by complex transcriptional networks in *C. albicans* (Ene and Brown 2014; Askew et al. 2009). In a certain way, metabolic adaptation within host-niches is linked to all other virulence factors of the fungus in these niches and metabolic adaptation is integral to *C. albicans* commensalism and pathogenicity (Brown et al. 2014a).

1.2.1. Integration of Carbon Metabolism with Virulence

The generation of new biomass is manly obtained by carbon assimilation (i.e. growth). So, in the immunocompromised host the rapid growth of *C. albicans* depends on the efficient uptake of available carbon sources in vivo. Moreover, the fungal cells must adapt its metabolic programme as it encounters new microenvironments in the host. Various studies have indicated that the regulation of carbon metabolism is intimately linked to the control of virulence in *C. albicans* (Nowrousian 2014; Ene and Brown 2014) (Figure 7).

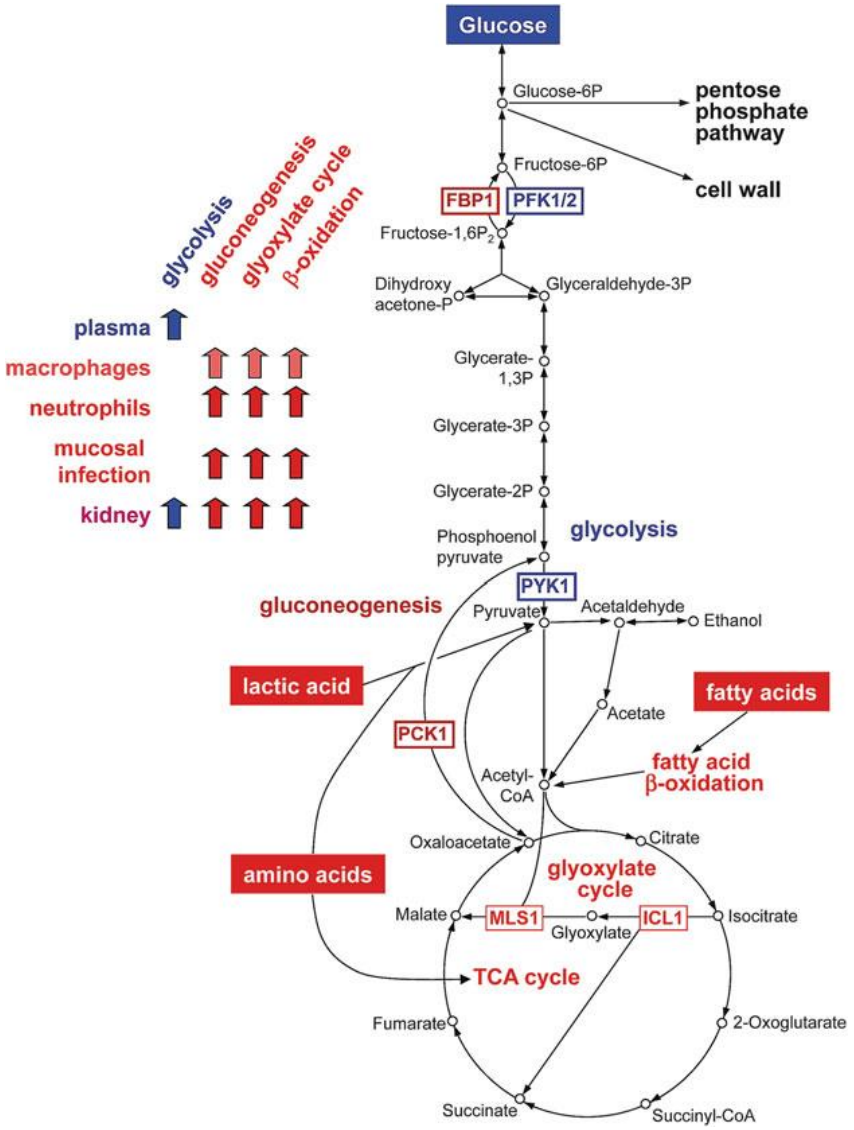

Figure 7. Central carbon metabolism in *Candida albicans* cells occupying different host niches. The right side of the figure indicates central carbon metabolism, with the main pathways highlighted. Glycolysis and glucose are highlighted in blue. Gluconeogenesis, the glyoxylate and tricarboxylic acid (TCA) cycles, and fatty acid b-oxidation are highlighted in red, as are the alternative carbon sources lactate, amino acids and fatty acids. Adapted from (Ene and Brown 2014)

1.2.1.1. Carbon adaptation modulates stress resistance

Metabolism is indirectly related with the virulence of *C. albicans* by improving stress adaptation to different microenvironments. Resistance to stress is required for *C. albicans* virulence promoting the increase of fungal cells survival in host-niches by reducing their vulnerability to local environmental stresses and to phagocytic killing (Brown et al. 2012; Arana et al. 2007; Patterson et al. 2013).

The response of *C. albicans* to environmental stress is dependent on the pre-adapted metabolic state of these fungal cells, and also upon the availability of nutrients encountered in host microenvironment. The analysis of *C. albicans* stress responses has been achieved on cells cultured on rich, glucose-containing media that differ significantly from host microenvironments which are often glucose-limited (Brown et al. 2012).

Recent data indicates dramatic effects upon the stress resistance of *C. albicans* by changing carbon source utilized (Ene et al. 2012a; Rodaki et al. 2009).

When *C. albicans* is transiently exposed to glucose some genes involved in oxidative stress adaptation are expressed, which will enhance cellular resistance to acute oxidative stress (Rodaki et al. 2009). This process is regulated by glucose-sensing pathways and possibly reflects the adaptive prediction (Mitchell et al. 2009), whereby *C. albicans* has 'learnt' over evolutionary time to anticipate the attack of phagocytic cells following entry to the bloodstream (Brown et al. 2014b).

Resistance to osmotic stress and antifungal drugs is also affected by carbon adaptation (Ene et al. 2012a; Ene et al. 2012b). These effects are mediated partly through PKA signaling and may relate to metabolic adaptation upon cellular abundances of osmolytes such as glycerol, and antioxidants such as glutathione and trehalose (Rodaki et al. 2009; Gonzalez-Parraga et al. 2003; Gonzalez-Parraga et al. 2010; Giacometti et al. 2009). For example, some short metabolic branches of the glycolytic pathway are used for synthesize glycerol and trehalose. Though, these effects can also be involved in cell wall remodeling of *C. albicans* (Ene et al. 2012b; Ene et al. 2012a). Growth on different carbon sources promotes changes in *C. albicans* cell wall both architecturally and biophysically. In this sense, the cell wall of *C. albicans* suffered a pre-adaptation, which confers to *C. albicans* cells different abilities to survive the imposition of osmotic and cell wall stresses (Brown et al. 2014a).

Recent studies comparing cells grown on glucose and on lactate showed that yeast cells grown on lactate presented higher resistance to osmotic stress, amphotericin B, and caspofungin, and reduced resistance to an azole antifungal drug (Miconazole) (Ene et al. 2012a). These studies

suggest that the metabolic adaptation of *C. albicans* cells to the availability of nutrients encountered in host-niches have an impact in their ability to overcome local environmental stresses and resist antifungal drug therapy (Brown et al. 2014a).

1.2.1.2. Carbon adaptation triggers dynamic changes in the C. albicans cell wall

The dynamic and complex structure of polysaccharides and proteins that constitutes cell wall of *C. albicans* suffers remodeling of both composition and architecture depending not only on the growth phase but also on developmental stage and in response to various external signals and also in response to the carbon sources present in the surrounding media (Ene et al. 2012a; Ene et al. 2013; Bowman and Free 2006; Chaffin et al. 1998). As already described above the cell wall structure comprises a relatively thick inner matrix of β-glucan and chitin that is decorated with a dense coat of mannan fibrils (Gow and Hube 2012a; Hall and Gow 2013). These mannans represent heavily N- and O-glycosylated proteins, many of which are covalently crosslinked to the carbohydrate infrastructure (de Groot et al. 2004; Hall et al. 2013). This structure has been defined for C. albicans cells grown on glucose.

In fact, the assimilation of alternative carbon source in vitro, such as lactate, by *C. albicans* results in alterations in cell wall architecture, and ultimately changes in the host's immune responses (Ene et al. 2013; Ene et al. 2012b; Ene et al. 2012a). These data shows that *C. albicans* cell wall is extremely influenced by the cell's metabolic status, which is depending upon the availability of nutrients within host-niches. And so, this leads to the adjustment of host-pathogen interactions (Lee and Munro 2014).

The relative portions of cell wall components are alike for glucose- and lactate-grown cells (Brown et al. 2014a). Nevertheless, cell wall biomass is significantly reduced after growth on lactate, and the cell walls of lactate-grown cells are thicker than glucose-grown cells (Figure 8) (Ene et al. 2012a).

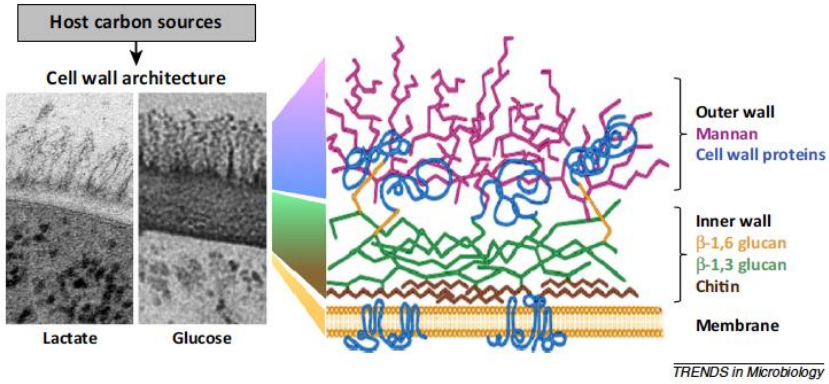

Figure 8. The impact of different carbon sources in cell wall architecture. Transmission electron micrographs of the *Candida albicans* cell wall from lactate-grown cells and glucose grown cells are shown on the left (Ene et al. 2012a). On the right, illustration of the structure of the *C. albicans* cell wall. Reviewed and adapted form (Brown et al. 2014a).

These dynamic changes have still not been described; perhaps depend upon the construction of new cell wall. The mechanisms by which carbon source influences *C. albicans* cell wall architecture remains unclear, but it seems to involve a combination of metabolism and signaling (Brown et al. 2014a).

Regardless of the mechanisms by which they occur, the effects of carbon source upon cell wall architecture that have been proved in vitro seems to be of extreme relevance in host niches because these changes in cell wall structure were observed in blood cultivation and under vaginal simulating conditions (Sosinska et al. 2008; Lowman et al. 2011). Thus, in host-niches the available carbon sources must have a strong effect on the architecture and functionality of the *C. albicans* cell wall (Brown et al. 2014a).

1.2.1.3. Metabolic adaptation influences virulence factors

In addition to promoting cell wall remodeling and stress resistance there are some evidences in longstanding reports that showed influences of metabolic adaptation in pathogenicity of *C. albicans* by modulating the expression of key virulence features (Figure 9) (Brown et al. 2014a).

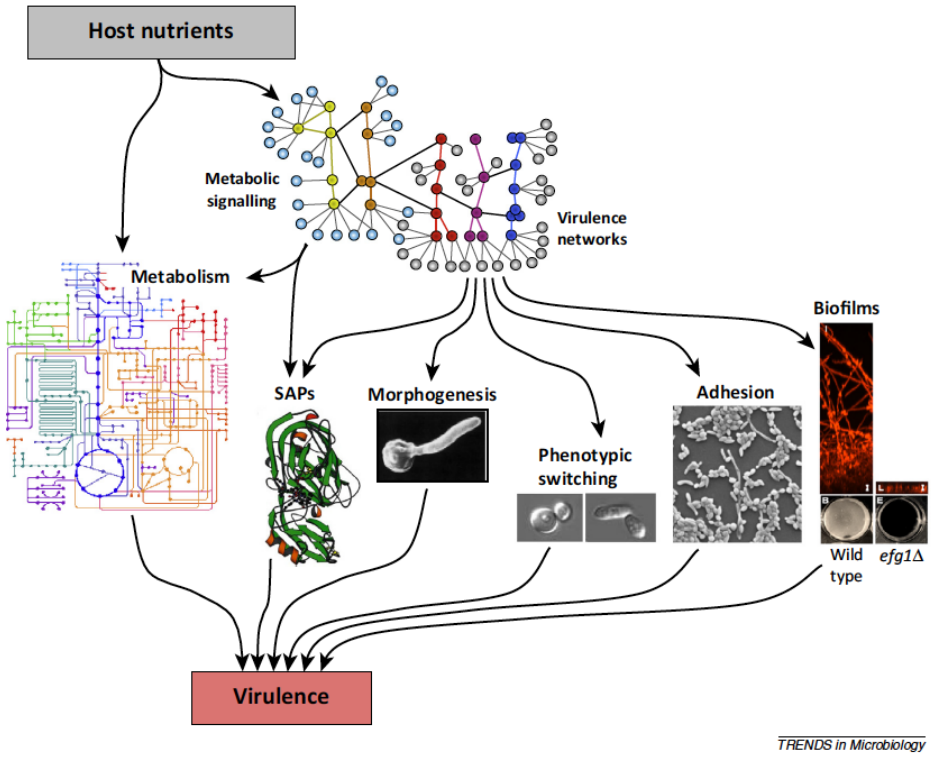

Figure 9. Nutrients influence the expression of key virulence factors in *Candida albicans*. Adapted from (Brown et al. 2014a)

For instance, glucose is one of the many stimuli that can induce hyphal morphogenesis, and glycolytic genes are expressed during the transition between yeast budding form to hyphal form (Hudson et al. 2004; Maidan et al. 2005). During the white–opaque phenotypic switch, the white cells up-regulate glycolytic genes while opaque cells up-regulate genes involved in respiratory metabolism (Lan et al. 2002). It is known that the expression of SAPs is regulated in response to available nitrogen and carbon sources (Hube et al. 1994). For example, SAP2 is expressed at high levels when *C. albicans* cells are grown on glycerol, at medium levels on glucose or galactose, and at low levels when grown on ethanol (Hube et al. 1994). The ability of adhesion and biofilm formation of *C. albicans* cells is also influenced by the growth on different dietary sugars (Samaranayake and MacFarlane 1982; Critchley and Douglas 1985; Jin et al. 2004).

This metabolic adaptation seems to be actively managed with the regulation of important virulence factors via complex networks. For example, carbon metabolism (in part via Tye7) as well as yeast–hypha morphogenesis, white–opaque phenotypic switching, and stress resistance are controlled by adenylyl cyclase–PKA–Efg1 signaling axis (Giacometti et al. 2009; Doedt et al. 2004; Harcus et al. 2004; Zordan et al. 2007; Morschhauser 2010). The reinforcement of the idea that metabolism actively modulates other *C. albicans* virulence attributes is supported by at least two essential observations: (1) mutations that disrupt key aspects of metabolism also affect cell wall integrity, stress sensitivity, virulence factors, and pathogenicity (Pande et al. 2013; Brega et al. 2004; Lorenz and Fink 2001; Martinez and Ljungdahl 2005; Noble et al. 2010; Dagley et al. 2011; Nobile et al. 2012); (2) regulatory networks that are required for GI colonisation or systemic infection include metabolic components (Perez et al. 2013).

The expression of virulence factors can also be indirectly influenced by metabolism, when a longer-term relationship between host-niches and pathogen are established. In the absence of glucose, for instance, *C. albicans* cells use amino acids as a carbon source, excreting the excess nitrogen in the form of ammonia, which results in the raises of ambient in pH of the local environment, thereby triggering hyphal development (Vylkova et al. 2011). This phenomenon might prevent the macrophage killing of *C. albicans* cells by inhibiting acidification of the phagolysosome, and promoting morphogenesis in other host niches (Losse et al. 2011).

1.2.1.4. Carbon adaptation modulates immune surveillance

The immune surveillance is also modulated by metabolic adaptation, which influences *C. albicans* pathogenicity. Phagocytic cells (primarily macrophages and neutrophils) are important in the prevention of *C. albicans* infection (Brown 2011). These cells of the innate immune system attempt to recognize *C. albicans* cells initially via PRRs that detect specific PAMPs on the fungal cell surface. These initial PAMP–PRR interactions activate phagocyte intracellular signaling pathways, for example via the Dectin-1/SYK/CARD9 and TLR4/TRIF–MYD88 pathways. This triggers induction of antimicrobial effector mechanisms such as the respiratory burst, as well as the release of a variety of pro-inflammatory cytokines, chemokines, and lipids that stimulate other leukocytes and attract them to the site of infection (Brown 2011).

Following phagocytosis process, the fungal cell is trapped within the phagosome, which then undergoes maturation and lysosomal fusion to create the phagolysosome (Fernandez-Arenas et al. 2009). The outcome of the fight between fungus and host, which depends upon numerous

individual skirmishes, has a major impact upon disease outcome. Thus, growth conditions that physiologically favor the fungus over the phagocytic cells may tip the balance, reducing phagocytic efficacy. The interaction between fungus and host immune cells is affected by the metabolic environment at the local of infection. *C. albicans* cells grown on lactate are less visible to the immune system than cells grown on glucose (Figure 10).

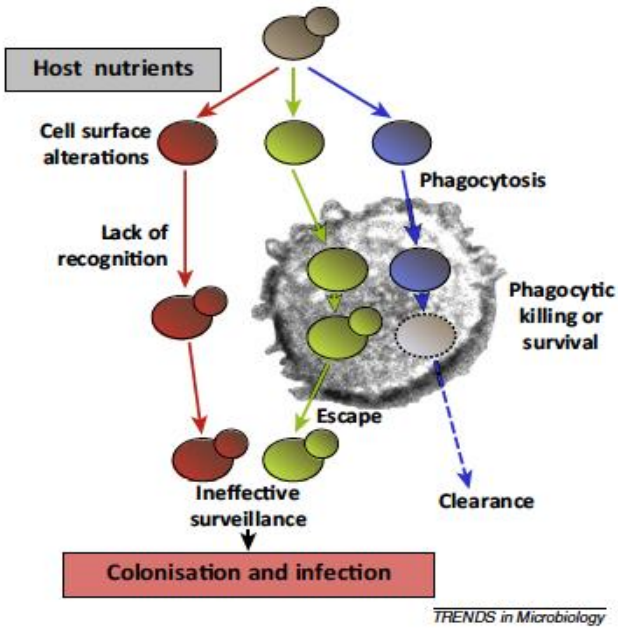

Figure 10. Changes in carbon source impact on immune surveillance. Alteration in the recognition of *Candida albicans* cells by innate immune cells and by reducing the susceptibility of the fungal cells to phagocytic killing via elevated oxidative stress resistance. Adapted from (Brown et al. 2014a)

Lactate-grown cells of *C. albicans* are less visible to the immune system than cells grown on glucose (Figure 10) (Ene et al. 2013). In the same study, Ene et al. observe that *C. albicans* cells grown on lactate stimulate the production of more interleukin-10 (IL-10) and less IL-17 by human peripheral blood mononuclear cells from healthy volunteers, than glucose-grown cells (Ene et al. 2013). It was also reported that lactate-grown *C. albicans* cells are also phagocytized less efficiently by murine macrophages and those lactate-grown *C. albicans* cells that are engulfed by the macrophages are better able to kill and escape from macrophages (Figure 10)(Ene et al. 2013). These interesting reports suggest that growth on a non-fermentable secondary carbon source confers reduced visibility of *C. albicans* cells to the immune system, and are less easy to kill than glucose-grown cells (Ene et al. 2013). This reduced immune visibility of lactate-grown *C. albicans* cells probably is due to the changes that cell wall experience in that microenvironment. Many of the key PAMPs involved in immune recognition are located in the cell surface wall, and

changes in cell wall structural components are known to affect immune recognition (Lowman et al. 2011; Lewis et al. 2012; Netea et al. 2006; Adams et al. 2008; Lowman et al. 2014). Further observations link metabolic adaptation with cell wall structure and immune recognition. The clearance of *C. albicans* cells during systemic infection is mediated by Dectin-1, and differences in that receptors have been shown to relate to the differential activation of cell wall biosynthetic functions in vivo within renal microenvironments (Marakalala et al. 2013).

A crucial role was recently proposed for glucose metabolism in the activation of immune cells. This role lies on the 'Warburg effect', which means that in the normal way naïve and resting cells metabolize glucose mainly via oxidative phosphorylation but switching to aerobic glycolysis ('Warburg effect') is crucial for proliferating lymphocytes (Delgoffe et al. 2011), and subsequently for important anti-*Candida* mechanisms such as the deployment of a T helper 17 (Th17) response (Kim et al. 2013). Furthermore, recent studies have shown that glycolysis and succinate play key roles in modulating the capacity of the innate immune system to mount a proper inflammatory response (Tannahill et al. 2013).

A recent hypothesis has come up that glucose also may have impact in modulation of antifungal host defense (Moyes et al. 2014). This hypothesis, which needs further study, has been recently reinforced by the observation that in epithelial cells mTOR (a key regulator that integrates nutrient inputs and energy levels to control cell growth and proliferation) is central for protection against *C. albicans*-induced cell damage (Moyes et al. 2014). Another example of metabolic modulation of immune responses is provided by endogenous tryptophan catabolism in the GI mucosa, which promotes IL-22 production by innate lymphoid cells, which in turn enhances intestinal immunity and protection against *C. albicans* (Zelante et al. 2013).

Altogether, the local metabolic environment of the host and the metabolic adaptation of *C. albicans* contribute to the efficacy or failure of local immune surveillance mechanisms (Brown et al. 2014a).

1.3. Objectives and outline of the thesis

The present work lies on previous studies that observed that *C. albicans* deleted in both copies of *RLM1* gene (*Δrlm1/Δrlm1*) showed typical cell wall weakening phenotypes, such as hypersensitivity to Congo Red, Calcofluor White and caspofungin, and that the cell wall of the mutant showed significant increase in chitin (213%) and reduction in mannans (60%), comparing with wild type (WT) strain (Delgado-Silva et al. 2014).

This and other studies of *C. albicans* stress adaptation have generally been performed on glucose-grown cells, leaving the effects of alternative carbon sources upon stress resistance largely unexplored. Thus, the main objective of this work is to understand the role of *RLM1* in the manifestation of *C. albicans* important virulence factors and in the interaction of with phagocytes under the influence of different carbon source, namely lactate..

First, the growth rate and growth metabolites were quantified in order to compare cells growth on glucose or lactate. Then, several virulence factors were evaluated, in cells grown on lactate or glucose, such as the ability to grow under cell wall damaging stress agents, filamentation, adhesion and biofilm formation. Finally, the involvement of *RLM1* in host-pathogen interaction was evaluated by using murine macrophage-like cell line J774, with cells adapted to different carbon source, lactate and glucose.

The work presented was developed in the Centre of Molecular and Environmental Biology (CBMA) University of Minho, Braga, Portugal.

Methods and Materials

Carbon source impacts in *C. albicans* virulence factors

Strains and growth conditions

Quantification of metabolites produced during *C. albicans* growth

Susceptibility assays

Filamentation tests

Adhesion assay and biofilm formation

Secreted Aspartyl Proteinases production

Gene expression analysis

Carbon source impacts in *C. albicans* Immune Recognition

Macrophage cell cultures

Macrophage-killing assay

Determination of lactate dehydrogenase activity and cytokines measurement

Statistical analyses

2. Methods and Materials

2.1. Carbon source impacts in *C. albicans* virulence factors

2.1.1. Strains and growth conditions

Five *Candida albicans* strains were used during this study, the wild-type strain SC5314 from (Gillum et al. 1984), two *RLM1* mutant strains, SCRLM1M4A and SCRLM1M4B (*Δrlm1/Δrlm1*) (Delgado-Silva et al. 2014) and two *RLM1* complemented strains, SCRLM1K2A and SCRLM1K2B (*Δrlm1/Δrlm1+RLM1*) (Delgado-Silva et al. 2014). All strains were stored as frozen stocks with 30% (v/v) glycerol and cultured onto yeast exctract-peptone-dextrose (YPD) agar plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose and 2% w/v agar) at 30°C.

For all the experiments, yeast cells were grown in minimal medium containing a carbon source (2% glucose or 2% lactate) and 0.67% yeast nitrogen base without amino acids (YNB) at a pH 5.2 to 5.6 and at 30°C.

Growth curves

Initially, it is important to understand how the strains used in this study growth in the conditions here presented. So, this study began by monitoring the growth of all the strains in the conditions above described. After that, all the following procedures in this study were conducted under the knowledge obtained from this simple experiment.

2.1.2. Quantification of metabolites produced during *C. albicans* growth

In the meantime of the growth curves experiment, 1 mL of cells were harvested by centrifuged for 5 mins at 5000 g and the supernatant was analyzed by HPLC (Hitachi, Lacrome Elite Demo) with an organic acid column (phenomex). This approach will give some information of a few metabolites (specially TCA metabolites) that are been produced in the growth phase under the conditions above described.

2.1.3. Susceptibility assays

Fungal cells were incubated overnight in liquid YNB medium containing 2% of carbon source (glucose or lactate) at 30 °C, 200 rpm, and then diluted to OD600 = 1 with fresh medium. Drop tests were performed by spotting 5 µl of the serially diluted cell suspension onto YNB + 2% Glucose or 2% Lactate agar plates supplemented with the following compounds: 200 µg/ml calcofluor white (CFW) (Fluorescent Brightener M2R, Sigma), 100 µg/ml Congo Red (CR), 90 ng/ml caspofungin (CFG), 10 mM caffeine (Sigma), 0.035% (w/v) SDS (BDH Chemicals). Plates were incubated 48h at 30 °C before observation.

2.1.4. Filamentation tests

All strains were grown overnight in liquid minimal medium, YNB, containing 2% of carbon source (glucose or lactate) at 30 °C, 200 rpm. Filamentation was induced by diluted yeast cells to OD600 = 1 with Dulbecco's modified Eagle's medium (DMEM) and incubated at 37 °C and 5% CO_2. In order to observe the filamentation feature, yeast cells were labeled with calcofluor white (CFW) and monitored by fluorescence microscopy (Leica, DM5000B+CTR5000+ebq100) at 0, 30 min, 1h, 2h and 4h of incubation.

2.1.5. Adhesion assay and biofilm formation

To determine the impact of different carbon sources on adhesion and biofilm formation ability, 24-well microplate (Orange Scientific, Braine-l' Alleud, Belgium) were filled with *C. albicans* cell suspensions (1 mL containing $1x10^6$ cells/mL) grown on each carbon source-growth condition (2% Glucose or 2% Lactate) as described previously, and incubated at 37 °C and at 120 rpm.

Adhesion ability was measured after 2h of incubation and the biofilm formation ability was inspected after 24h and 48h. Concerning the 48h of incubation samples, an extra step was preformed, at 24h, 500 µL of cultured medium was removed and replaced by fresh medium.

The experiments were preformed in triplicate.

Adhesion assay and biofilm biomass assessment

Adhesion and biofilm formation feature was assessed trough quantification of total biomass by crystal violet (CV) staining (Stepanovic et al. 2000). For that, after the incubation times previously defined, the medium was totally aspired and non-adherent cells were removed by washing the wells with sterile water MiliQ. Considering total biomass quantification, firstly cells were fixed with 1

mL of methanol (100% v/v), which was removed after 15 min of contact. The microplates were allowed to dry at room temperature, and 1 mL of CV (1% v/v) was added to each well and incubated for 5 min. Then, the wells were gently washed twice with 1 mL of sterile MiliQ water and 1 mL of acetic acid (33% v/v) added to release and dissolve the stain. The solution with the dissolved stain was transferred to 96-well plate for absorbance measurement. The absorbance of the obtained solution was read in triplicate in a microplate reader (SpectraMax Plus) at 570 nm. Results were presented as absorbance per unit area of well ($Abs570nm/cm^2$). Statistical analysis was performed using GraphPad Prism 6 software for Macintosh. The results were compared using a two-way analysis of variance (ANOVA) with the Bonferroni pos-hoc test. All tests were performed with a 95% confidence level.

Biofilm structure

The biofilm structure was also observed by Scanning Electron Microscopy (SEM). For each conditions tested (glucose-grown cells and lactate-grown cells), biofilms were formed into 24-wells microplates containing clean sterile glass coverslips (Ø13 mm) (Orange Scientific, Braine-l' Alleud, Belgium), as elucidated above. The samples were fixed with 2.5% glutaraldehyde (Merk) for at least 24h at 4 °C. After that, the samples were washed carefully with PBS (pH 7) and the dehydrated with increasing percentages of ethanol (using 30, 50, 70, 80, 90, 100% (3x), each percentage was removed after 15 min of contact) and air dried for additional 20 min. Samples were kept in a desiccator until the base of the wells were mounted onto aluminum stubs, sputter-coated with gold and observed with an NanoSEM - FEI Nova 200 (Azurém, Guimarães, Portugal).

2.1.6. Secreted Aspartyl Proteinases production

The enzymatic activity can be detected by the formation of an opaque halo of protein degradation around the colonies grown on a specific agar plate, according to (Ruchel et al. 1982) To analyze the protease activity, cells suspensions grown as described previously, were cultured onto protease agar medium composed by 2% (w/v) agar, 1.17% (w/v) yeast carbon base, 0.01% (w/v) yeast extract and 0.2% (w/v) bovine serum albumin (BSA), at pH 5.0. Then, the plates were incubated at 30 °C for a period of 5 days. In order to guarantee the influence of the carbon source, secreted proteinases were also evaluated in agar plates, taking into consideration the carbon source (Barros et al. 2008). For that, all *C. albicans* strains were tested in BSA-Agar medium containing 0,2% (w/v) BSA, 1.45% (w/v) YNB (Difco Laboratories, Detroit, MI, USA), 2%

(w/v) Glucose or 2% (v/v) Lactate and 2% (w/v) agar, at pH 5.0. The plates were incubated in the same condition above described.

After incubation, each plate was placed in contact with 15 mL of dye solution (0.1% (w/v) amido black, 30% (v/v) methanol, 10% (v/v) acetic acid and 60% ultra-pure water) for 1h. Afterwards, the plates were washed twice with acetic acid (33% v/v) and dried at room temperature.

2.1.7. Gene expression analysis

Gene selection for quantitative Real-Time PCR

Eight genes (*ALS3*, *HWP1*, *AGP2*, *PUT2*, *GCV2*, *CIT1*, *SOU1* and *GDP1*) were selected to analyze their expression in the different growth conditions, using glucose or lactate as carbon source. Actin (ACT1) gene was selected as a reference *Candida1* housekeeping gene (Delgado-Silva et al. 2014). These genes were selected based on a previous work of our group (Delgado-Silva et al. 2014) and so the primers for quantitative Real-Time PCR (qRT-PCR) were also previous designed for that work. In this work, the same primers were used but in the context of the conditions that has been described along this work.

Table 1. Primers used in this study.

Primers	Sequence (5'- 3')
ALS3	Fw: CGTCCATTTGTTGACGCTTA
ALS3	Rev: GCGGTTAGGATCGAATGGTA
HWP1	Fw: TCTACTGCTCCAGCCACTGA
HWP1	Rev: CCAGCAGGAATTGTTTCCAT
AGP2	Fw: TGTGGCTATGCAGAACTTGG
AGP2	Rev: AGACAGGAACCCCATGACTG
PUT2	Fw: TTCTCCTGGTGTTTGGAACC
PUT2	Rev: TAATGCGGCTGTAGCAGATG
GCV2	Fw: TGGGTGCTGATGTTTGTCAT
GCV2	Rev: AGCTTGTGCTCCCAACATCT
CIT1	Fw: CCACGAAGGTGGTAACGTCT
CIT1	Rev: TTTTTCAATGGCTTCCTTGG
GPD1	Fw: TTGCTCGTGCTAAATGGTCA
GPD1	Rev: CACCCCAACCTAAACCTT

[a] Restriction sites introduced into primers are underlined.

Yeast cells preparation

An inoculum of each yeast strain, obtained from YPD plates, was resuspended in 100 mL of YNB with 2% Glucose or 2% Lactate and incubated at 30 °C, under agitation at 200 rpm. Reached late exponential growth phase, up to $3x10^8$ cells were harvested by centrifugation at >12000 g for 2 min and the supernatant was discarded and the pellet cells stored at -80° C, until use.

RNA extraction

Ribonucleic acid (RNA) extraction was performed using Ambion RiboPure™-Yeast Kit (Life Technologies Corporation, Carlsbad, USA). The RiboPure-Yeast method disrupts yeast cell walls by beating cells mixed with an aqueous lysis buffer, SDS, phenol and 0.5 mm Zirconia Beads on a vortex adaptor for 10 min. Before RNA extraction, it was dispensed 750 μL Zirconia Beads were dispensed 1.5 mL screw cap tube for each sample. The collected cells were resuspended in lysis reagents (480 μL lysis buffer, 48 μL 10% SDS and 480 μL Phenol:Chloroform:IAA) and vigorously vortexed for 10-15 seconds. The resulting mixtures (cells and lysis reagents) were transferred to one of the prepared tubes containing 750 μL cold Zirconia Beads and vortexed for 10 mins. The lysate is centrifuged for 5 min at 16000 g at room temperature to separate the aqueous phase, which contains the RNA, from the lower organic phase, which contains proteins, polysaccharides, and other cellular fragments.

After colleting the aqueous phase (containing RNA), the Ambion RiboPure™-Yeast Kit was used for further RNA extraction and purification according to the manufacturer's recommended protocol. To avoid residual DNA, samples were treated with Ambion DNA*free* ™ reagents, and DNAse enzyme, included in the kit.

Synthesis of cDNA

cDNA was synthesized using iScript Reverse Transcrption Supermix kit (Bio-Rad) according to the manufacturer's instructions. The primers used for cDNA synthesis were the ones already referred above. Real time PCR using SYBR green technology was performed on CFX96 real-time detection system in 96-well microtitre plates using a final volume of 20 μl (Bio-Rad). SYBR Green Supermix (Bio-Rad and Applied Biosystems) and 0.1 μM of primers were mixed with DNAse and RNase-free water to make the 9/10th of the total reaction volume and 1/10th of cDNA was added into the mixture. The following amplification program was used: 3 min of denaturation and polymerase activation at 95°C, 40 cycles of real time PCR with 2-step amplification were

performed consisting of 15s at 95°C for denaturation, 45s at 60°C for annealing. All samples were amplified in duplicate and the mean was obtained for further calculation.

Statistical analysis

Gene expression was normalized to the housekeeping gene ACT1 and analyzed by using the comparative threshold cycle (ΔΔCT) method. Data was presented as the fold difference in expression relative to wild-type (WT) gene expression.

2.2. Carbon source impacts in *C. albicans* Immune Recognition

2.2.1. Macrophage cell cultures

The murine macrophage-like cell line J774A. 1 (American Type Culture Center number TIB 67 (Ralph and Nakoinz, 1975)), was routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza Group, Ltd., Braine l'Alleud, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FBS; Biosera, Ringmer, United Kingdom), 1% glutamine, 1% sodium pyruvate, and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in tissue culture flasks (Nagle Nunc, Int., Hereford, United Kingdom) 5% (v/v) CO2 at 37 °C.

Sub-cultures were performed into new cell-culture flasks every 2 days. After confluent growth, macrophage cells were recovered and washed. Viable cells were determined by Trypan blue exclusion counting with the hemocytometer, and resuspended in DMEM to a final concentration of $4x10^5$ cells/mL.

2.2.2. Macrophage-killing assay

The macrophage-killing assay was preformed as previously described by Mckenzie (McKenzie et al. 2010) and under the same growth conditions described above. Macrophages and yeast cells were cultured in 96-well tissue culture plate (SpectraMax Plus) and incubated for 1h at MOI 5Y:1M *C. albicans*/macrophage ratios. After incubation the 96-well tissue culture plate was centrifuged for 2 mins at 750 g and 80 µl of supernatant culture was transferred to a new 96-well microplate and stored at -80°C for further cytokine measurement. In the first 96-well plate the final volume was restored by adding 80 µL of 10% saponin followed by gently up and down pipetting (a few times) in

order to lyse macrophages and release the adherent cells. Serial 10-fold dilutions were then plated YPD agar plate and incubated at 30 °C for 24h.

Data were obtained in triplicate from at least two separate experiments and presented as percentages.

2.2.3. Determination of lactate dehydrogenase activity and cytokines measurement

LDH

LDH leakage assay is one of the most used methods for cell viability determination due to its reliability, fastness and simplicity. Lactate dehydrogenase (LDH) is an intracellular enzyme existent in most eukaryotic cells, which is only released into culture medium upon cell death, or plasma membrane damage. Thus, LDH was measured in the supernatant of macrophages:yeast incubation to assess macrophage cell damage. LDH reduces pyruvate to lactate, coupled to the oxidation of NADH to NAD^+. This reaction can be followed spectrophotometrically at 340 nm (Korzeniewski and Callewaert 1983).

The collected samples were analysed in a 96 multiwell plate, each well containing: 40 µl of extracellular LDH sample and 250 µl of NADH (final concentration 0.28 mM). Reactions were started by adding 10 µl of pyruvate (final concentration 0.32 mM) to each well. Both NADH and pyruvate solutions were prepared in 0.05 M phosphate buffer pH 7.4. NADH conversion to NAD^+ was spectrophometrically evaluated in a microplate reader (Molecular Devices – SPECTRAmax Plus 384) at 340 nm, every 10 seconds for 3 minutes, at 30°C. The % of viability was calculated according to equation 1 (Holder et al. 2012).

$$\%viability = \left(1 - \frac{m_{sample} - \phi_{\min}}{\phi_{\max} - \phi_{\min}}\right) \times 100$$

(1)

Where m_{sample} is the LDH released into the supernatant from the wells containing the material; $\emptyset_{min}$ the LDH released into the supernatant from the wells containing only cells, and $\emptyset_{max}$ is the LDH released into the supernatant from the wells in which the cells were deliberately with DMSO:Ethanol (1:1), representing the maximum expected LDH value.

TNF-α and IL-10

Production of TNF-α and IL-10 by macrophages co-incubated with *C. albicans* SC5314, SCRLM1M4A, SCRLM1M4B, SCRLM1K2A AND SCRLM1K2B strains were quantified using a commercially available sandwich ELISA kit (Quantikine; R&D Systems, Abingdon, U.K.), (KMC 0102, Biosource, Camarillo, CA), respectively, and according to the manufacturer's instructions. The macrophage incubation medium used for TNF-α and IL-10 quantification was the same used in the LDH analysis. Determinations of LDH, TNF-α and IL-10 were carried out in triplicate, and the results are expressed as the mean of these results.

2.2.4. Statistical analyses

Results from at least two independent sets of experiments are expressed as means ± SD by Two-way (ANOVA), Bonferroni post-tes (Graphpad Prism 6) were used to determine statistical significance. The level of significance was set at a corrected P value of < 0.05.

Results and discussion

Identification of some metabolites during *C. albicans*

Impact of carbon source in *RLM1* mutant response to cell wall stress agents

Filamentation tests

Adhesion and biofilm formation

Secreted Aspartyl Proteinases production (SAPs)

Influence of carbon source *RML1*-responsive genes

Host-pathogen interaction: Participation of *RLM1* in Macrophage-*Candida albicans* interaction under the influence of different carbon source

3. Results and Discussion

Clearly, to cause infection *C. albicans* must grow and divide in diverse niches. Cells must assimilate locally available carbon sources that can include fermentable sugars and non-fermentable carbon sources (Lorenz and Fink 2001; Lorenz et al. 2004; Piekarska et al. 2006; Ueno et al. 2011; Vieira et al. 2010). Physiologically relevant sugars include glucose, galactose, and fructose among the others. But, in many hosts niches these sugars are only present at low levels and are even absent in the host. Therefore, other non-fermentable carbon sources become important to sustain yeast growth and its metabolism *in vivo* (Ueno et al. 2011; Vieira et al. 2010; Piekarska et al. 2006). These alternative carbon sources include amino acids and organic acids. A good example is lactate, which is present in ingested foods, produced via host metabolic activity, generated by endogenous lactic acid bacteria in the urogenital and gastrointestinal tracts, and is an important carbon source for *Candida* in the intestine (Ueno et al. 2011; Ene et al. 2012a).

So, before all the experiments proposed in this study, all the strains used were assessed for grown on YNB liquid medium with glucose or with lactate, at 30°C. Previous results of our group (Delgado-Silva et al. 2014), demonstrated that the growth rate of *C. albicans Δrlm1/Δrlm1* mutant was unaffected in YPD at 30° C. However it was important to assure the growth of these same strains grown in minimal medium with glucose or lactate.

In fact, the results showed that the growth rate of *C. albicans* strains was unaffected in minimal medium with glucose as observed by Delgado-Silva in YPD at 30° C (Figure 11). Similarly to glucose-grown *C. albicans* cells the growth rate of *C. albicans Δrlm1/Δrlm1* mutant was also not significantly affected, however the growth rate was significantly lower.

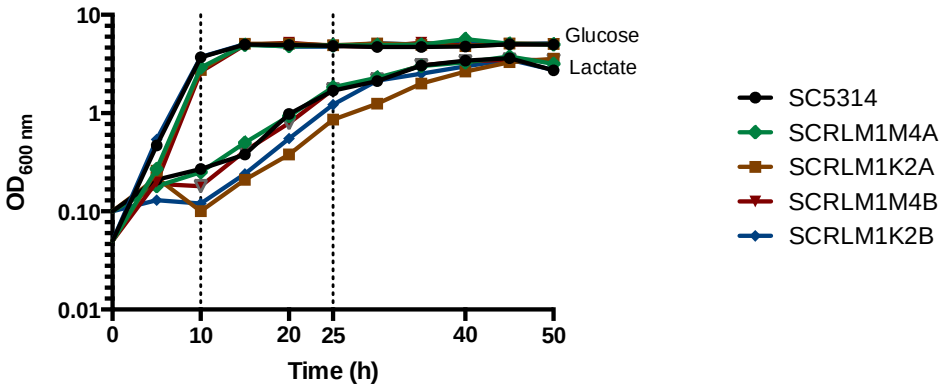

Figure 11. Growth curve of *C. albicans*. SC5314 (wt; black circles), homozygous mutant SCRLM1M4A and SCRLM1M4B (*Δrlm1/Δrlm1*); green diamond; red triangles) and complemented SCRLM1K2A and SCRLM1K2B (*Δrlm1/Δrlm1+RLM1*; brown squares; blue diamond).

3.1. Identification of some metabolites during *C. albicans*

During *C. albicans* strains growth samples were taken and analyzed by HPLC in order to evaluate glucose or lactate consumption and determine the presence of known metabolic metabolites produced. All strains were grown in conditions described before. In the Figure 12 the metabolites identified during glucose-grown cells and lactate-grown cells are presented.

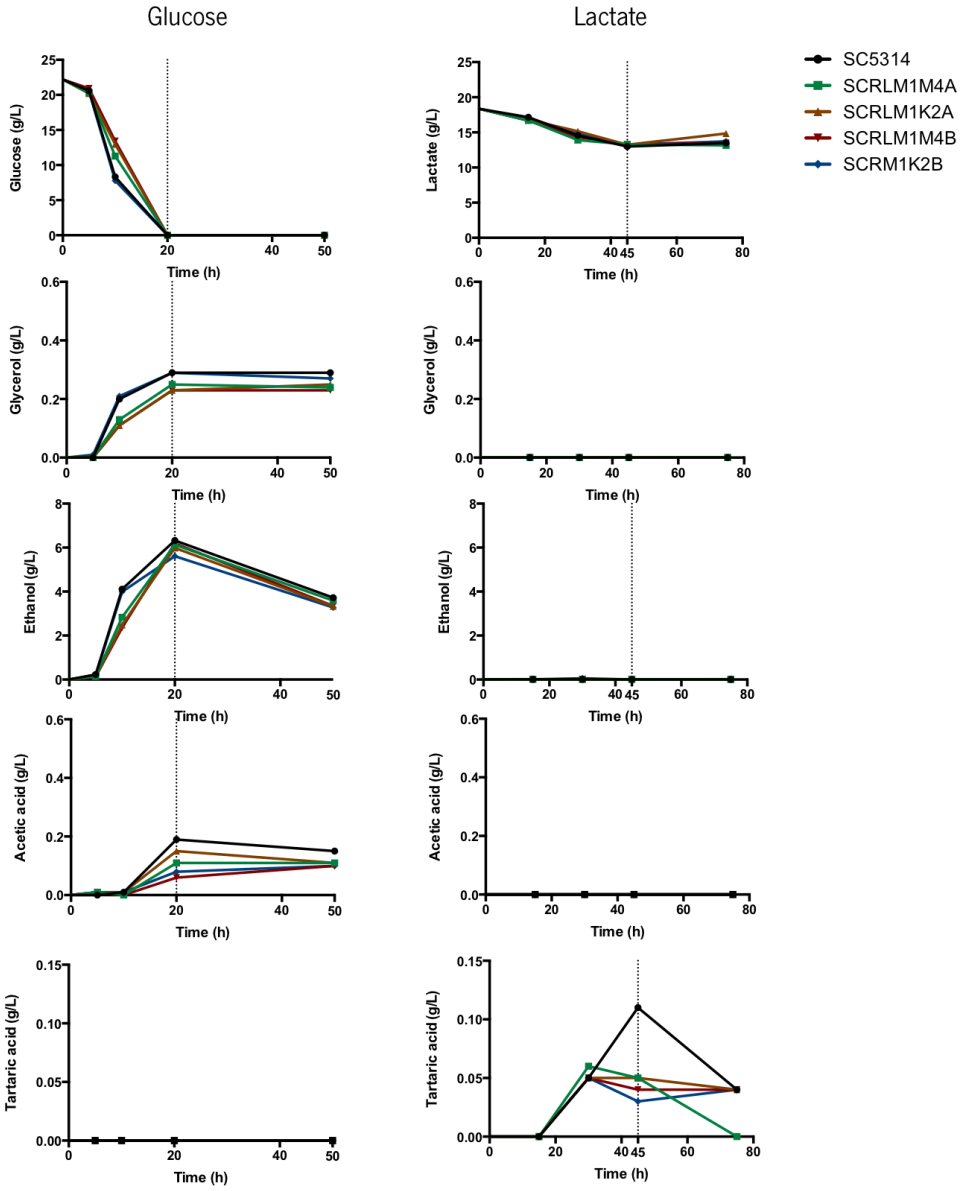

Figure 12. Identification by HPLC of metabolites during *C. albicans* growth on different carbon sources.

In glucose-grown cells it was possible to observe that during the first 20h of growth, glucose is totally consumed. During this period, some compounds are produced such glycerol, ethanol and acetic acid. It also interesting to note that when glucose is totally consumed the production of glycerol stabilizes while ethanol decreases for all strains tested. Curiously, the profile for acetic acid is slightly different since it decreases for the WT and complemented strains while for the mutant

this it stabilizes or increases. These results might suggest that *RLM1* can interfere with the shift of ethanol to acetic acid. For lactate-growns cells, the rate of consumption of lactate was slower than the rate of consumption of glucose, and after 45h of growth lactate consumption stabilized. Curiously, the yeast cells were unable to completely consume lactate, only around 32% of the initial lactate was consumed, contrary to glucose-grown cells that consume 100% of the initial glucose. During lactate growth, the ethanol produced was residual, and no acetic acid or glycerol was detected. Curiously, in lactate grown cells a relatively small mount of tartaric acid was observed, contrary to glucose grown cells, after 18h of growth that accumulated until 45h, the same time that lactate consumption ended, and then stabilized or diminished. No significant difference the amount of this acid was observed between the different strains. This experiments point to different metabolic route used by cells grown in the different carbon source. Although these results needs confirmation it indicated us that for lactate-grown cells other HPLC columns may be needed in order to identify compounds of that metabolic pathway.

3.2. Impact of carbon source in *RLM1* mutant response to cell wall stress agents

Previous study of our group determined that *RLM1* of *C. albicans* is involved in the CWI pathway, as described for *S. cerevisiae*, by comparing the sensitivity of the constructed mutant strains against a range of cell wall-perturbing agents as well as to agents known to be associated with altered cell walls (Delgado-Silva et al. 2014). In this study we aim to understand the impact that different carbon sources may have in cell wall integrity. It is important refer that all the information known, until now, about *RLM1* gene was obtained exclusively by *Candida* cells grown in YPD (glucose, as primary carbon source). As represented in Figure 13, the absence of a functional *RLM1* in *C. albicans* cells grown on glucose results in hypersensitivity to Congo red (CR) as described by Delgado-Silva (21014) but the hypersensitivity to Calcofluor White (CFW) reported by Delgado-Silva et. al (2014) was not observed in this work (Delgado-Silva et al. 2014).

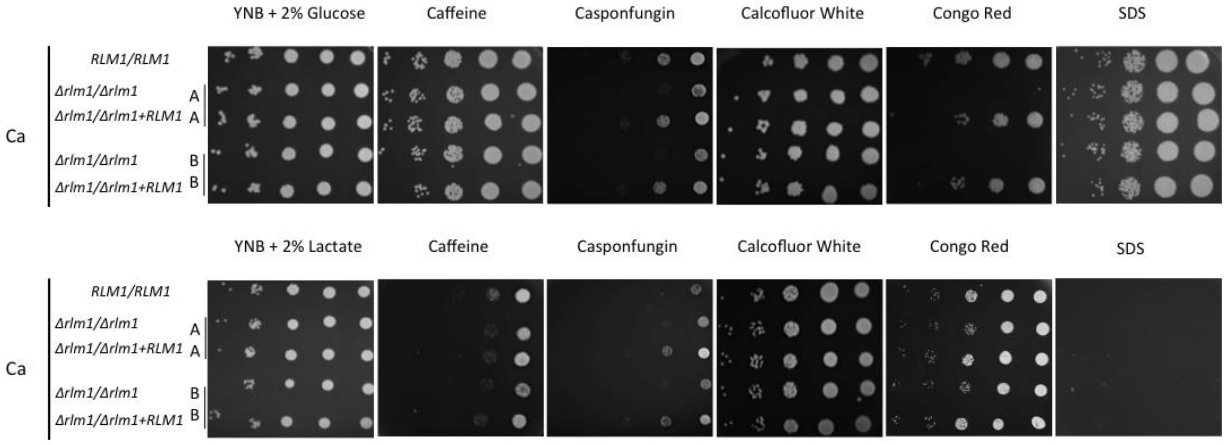

Figure 13. Sensitivity of *C.albicans* strains against agents that affect cell integrity. Serial 10-fold dilutions of YNB Glucose an YNB Lactate overnight cultures were spotted on YNB (2% Glucose or 2% Lactate) plates with 100μg/ml Congo red, 200μg/ml calcofluor white, 90ng/ml caspofungin, 10mM caffeine, 0.035% SDS. After incubation for 2 days at 30°C plates were photographed. *C. albicans* strains obtained from SC5314 (*RLM1/RLM1*) were two independently constructed homozygous mutant strains (*Δrlm1/Δrlm1*) SCRLM1M4A and SCRLM1M4B, and two independently constructed complemented strains (*Δrlm1/Δrlm1+RLM1*) SCRLM1K2A and SCRLM1K2B.

Additionally, in yeast cells grown in glucose the SC5314-derived *Δrlm1/Δrlm1* mutants were also more sensitive than the complemented and parental strains to the presence of Caspofungin (CFG). These cells (*Δrlm1/Δrlm1* mutant) were equally resistant to caffeine and unaffected by SDS as the WT and complemented strains.

On the other hand lactate-grown cells present hypersensitivity to caffeine and SDS, regardless of *RLM1* mutation, particularly to SDS in which none of the strains were able to grow. The absence of a functional *RLM1* in *C. albicans* cells grown on lactate results in hypersensitivity to Congo red (CR) and to Caspofungin, as observed in glucose-grown cells. However, cells grown on lactate appear to be more resistant to Congo Red than cells grown on glucose. In lactate-grown cells no differences were also observed in the sensitivity to CFW, as observed for glucose-grown cell.

It has been described that in response to the weakening of the cell wall, an increase in the cell wall chitin content is observed after activation of the so-called ''compensatory mechanism'' (Lagorce et al. 2003) However, Delgado-Silva et. al observed that under no stress condition, in the absence of a functional Rlm1p, *C. albicans* cell wall presents a different polymer organization which involves the increase of chitin content and decrease in mannans but does not seem to involve alterations in the β-1,3-glucans layer (Delgado-Silva et al. 2014).

The hypersensitivity observed in *C. albicans Δrlm1/Δrlm1* mutant cells to CR, which affects the assembly of β-1,3-glucan, and to CFG, an inhibitor of β-1,3-glucan synthase, may suggest an alteration in the organization of β-1,3-glucan, since the Delgado-Silva (2014) reported no alteration

in the glucan contents in the cell wall of *C. albicans Δrlm1/Δrlm1* mutant grown on glucose. In this case, the two-fold decrease in the cell wall mannans and therefore mannoproteins in the cell wall surface observed in the mutant in comparison with the wild-type by Delgado-Silva (2014), suggests that CR may access more easily to the glucans layer and affect more marked its assembly. Changes in the association between the polysaccharides, particularly the amount of mannoproteins, have also been described in response to cell wall weakening (Lagorce et al. 2003).

These results are in agreement with previous study that suggests that *C. albicans RLM1* is indeed involved in cell wall biogenesis and in cell wall remodeling (Delgado-Silva et al. 2014).

Considering cells grown in the different carbon sources, the hypersensitivity to CFG, that enter the cell to inhibit of β-1,3-glucan synthesis affects cells equally, regardless of the carbon source. However, for CR, which affects the assembly of β-1,3-glucan outside the cell, the *C. albicans Δrlm1/Δrlm1* mutant grown in lactate appear to be much more resistant to CR than grown in glucose. Recently, new studies have illustrated that lactate-grown cells are affected in their cell wall architecture and stress responses, indicating that, although the amount of cell wall polysaccharides is not affect, the thickness of the chitin+glucan layer is significantly reduced in comparison with glucose-grown cells (Ene et al. 2012a; Ene et al. 2013). Although no information on the cell wall components of *C. albicans Δrlm1/Δrlm1* mutant grown on lactate is available, the fact that these cells are more resistant when grown on lactate may be due to either the increase on the mannoproteins layer, hampering the access to the target molecule or, the amount of the target molecule may be reduced, as suggested in the previous studies.

The alterations mediated by an alternative carbon source such as lactate (used in this study) may contribute for the differences in the sensitivity to caffeine and SDS, which is not observed when *Candida* cells are grown on glucose. SDS is known to disrupt and solubilize the plasma membrane and is thus not a cell wall-specific compound (Daher et al. 2011; Plaine et al. 2008). However lactate-grown cells present higher cell wall porosity (Ene et al. 2012a), which might facilitate the permeabilization of SDS and render the cell more susceptible (Daher et al. 2011). This alteration is not related to *RLM1* since all strains presented the same phenotype. The mechanism by which caffeine induces cell wall stress is not understood, but lactate-grown *C. albicans* were much more sensitive than glucose grown cells and this susceptibility was not related to *RLM1*. Curiously, lactate-grown cells do not produce detectable amounts of glycerol, which is known to be osmostabilizer and thus, could contribute to the reduced or complete absence of growth in caffeine and SDS, respectively.

Altogether, these results are consisting with the data reported by Delgado-Silva et. al (2014), which observed that the lack of *RLM1* in *C. albicans* significantly down-regulated genes involved in carbohydrate catabolism and up-regulated genes involved in the utilization of alternative carbon sources (Delgado-Silva et al. 2014)

3.3. Filamentation tests

A striking feature of *C. albicans* is the capacity to grow as budding yeast and as filamentous forms. The yeast-to-hypha transition contributes to the overall virulence of *C. albicans* and may even constitute a target for the development of antifungal drugs (Shareck and Belhumeur 2011).

In order to observe if filamentation of RLM1 mutants was affected by different carbon source, the cells pre-grown on glucose or lactate were then incubated with DMEM at 37°C and 5% CO_2, and photographed in different time points.

The results depicted in Figures 14 and 15 demonstrated that both lactate-grown cells and glucose-grown cells were able to filament. It is important to note that the filamentation feature was significantly not affect by the lack of a functional *RLM1* in both growth conditions, even though the wild type presents slight longer hyphae. Still, it seems that *C. albicans* cells grown on lactate the on set of filamentation is later than cells grown on glucose. Another observation that might be brought to light after these figure analysis (Figures 14 and 15) is that lactate-grown cells are more aggregated than glucose-grown cells. This last result might be important in the biofilm formation.

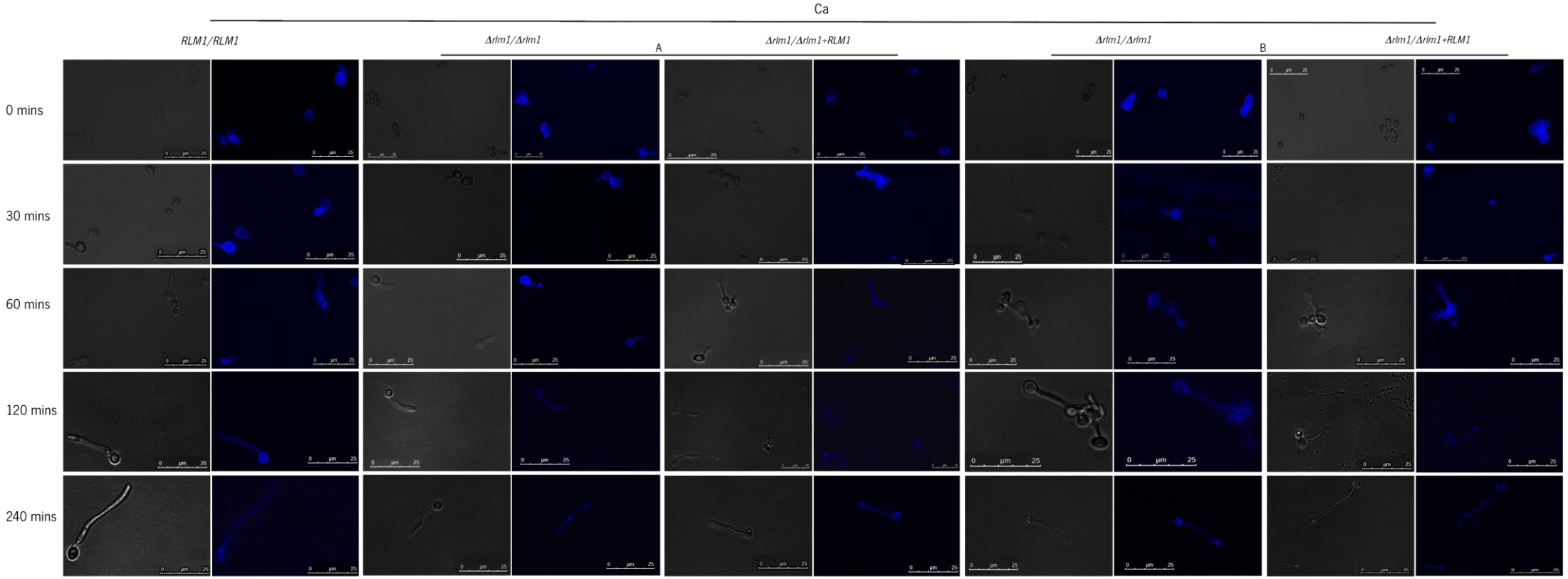

Figure 14. Filamentation of *C. albicans* glucose-grown cells. Evaluation of filamentation of *C. albicans* strains (SC5314, *RLM1* mutants and complemented) after supplemented with DMEM medium at 37 °C and monitoring for 30, 60, 120 and 240 minutes by fluorescence microscopy.

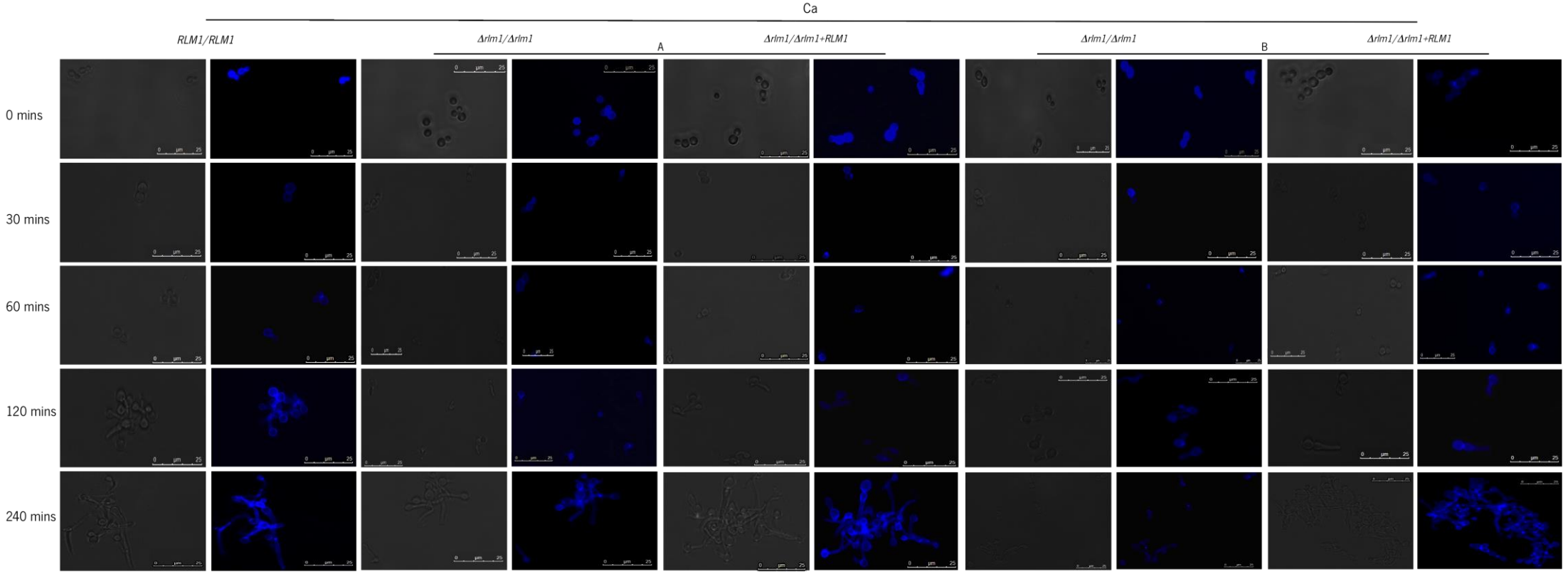

Figure 15. Filamentation of *C. albicans* on lactate as carbon source. Evaluation of filamentation of *C. albicans* strains (SC5314, *RLM1* mutants and complemented) after supplemented with DMEM medium at 37 °C and monitoring for 30, 60, 120 and 240 minutes by fluorescence microscopy.

Based on previous studies of our group (Delgado-Silva et al. 2014; Nogueira 2008), the filamentation feature was not affected by absence of *RLM1*. The results obtained in this study were in accordance with those studies, in cells grown on glucose as well as in cells grown on lactate. However, the higher aggregation observed in lactate-grown cells might be due to overexpressed of adhesion molecules, which means that those cells displayed higher levels of self adhesion, which might influence, for example, the biofilm formation (Ene et al. 2013; Ene et al. 2012a; Ene and Brown 2014).

3.4. Adhesion and biofilm formation

Adhesins are thought to promote adherence to host tissue, biofilm formation and colonization (Hoyer et al. 2008; Nobile et al. 2008).

According to Delgado-Silva et. al (2014), *Δrlm1/Δrlm1* mutant shows higher up-regulation of proteins involved in adhesion and biofilm formation. Additionally, Ene et. al (2012), demonstrate that lactate-grown cells display higher adherence ability and biofilm formation than glucose-grown cells. Based on these two reports, *C. albicans* strains were tested regarding their ability to adhere to a polystyrene surface (within 2h) and to form biofilm after 24h and 48h on lactate and on glucose (Figure 16).

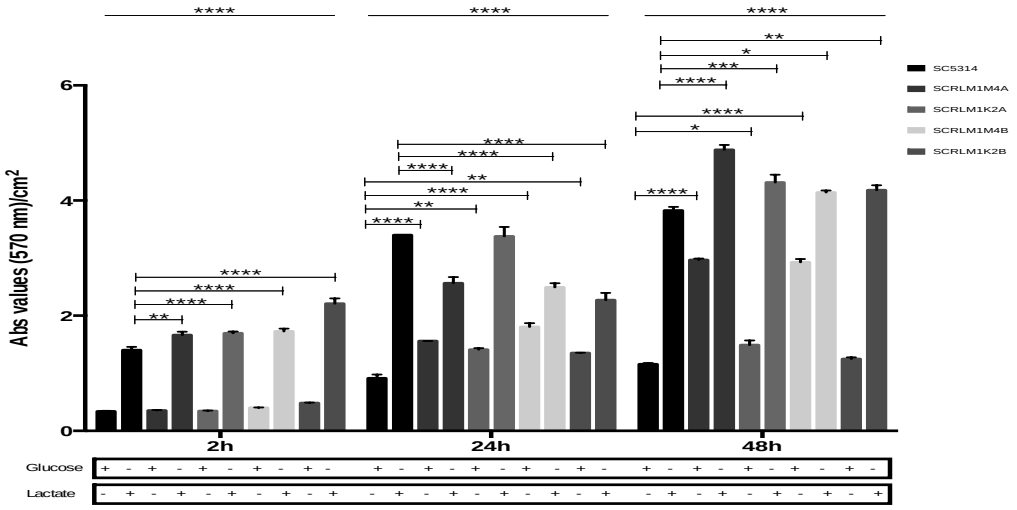

Figure 16. In vitro adhesion and biofilm formation. *C. albicans* cells from SC5314 (*RLM1/RLM1*), SCRLM1M4A and SCRLM1M4B (*Δrlm1/Δrlm1*) and complemented strains SCRLM1K2A and SCRLM1K2B (*Δrlm1/Δrlm1+RLM1*) were allowed to adhere (2h) and to form biofilm (24h and 48h) in polystyrene. The symbol * indicates that measurements were significantly different (P<0.005) from the WT strain and glucose as a primary carbon source.

Regarding adhesion after 2 hour on incubation, in glucose-grown cells no significance differences between strains were founded. In contrast, in lactate-grown cells the adhesion is overall mush higher for all strains ($P < 0.05$) but, as for glucose-grown cells, no clear trend was observed regarding the role of *RLM1* at this time point.

In the context of biofilm formation, a clear difference in total biomass formation was observed between glucose- and lactate-grown cells (24h and 48h). Overall, lactate-grown cells presented higher biomass than glucose-grown cells ($P < 0.05$). Additionally, at 24h the mutant strain presents different behaviors when grown on glucose or on lactate. On lactate-grown cells it seems that the mutant forms less biomass than the WT and complemented strains, which means that *RLM1* gene may influence biofilm formation. Although this effect does not appear to be consisting at 48h where *RLM1* mutant strains present higher biofilm formation in both carbon sources. An explanation for this delay in the biomass formation might be due adaptation of the mutant strain to lactate growth condition. The fact that *RLM1* mutants produced more biomass at 48h on glucose-grown cells was already described, but in lactose-grown cells no information was available. In general, the complemented strain, SCRLM1K2A and SCRLM1K2B, with some variations in its behavior, showed once more to be similar to WT. These variations in its behavior might be due to partial complementation of *RLM1* (Delgado-Silva et al. 2014). Together, these results indicate that *RLM1* acts as a negative regulator of in vitro biofilm formation, particularly when cells are grown on glucose.

The structure of the biofilm formation was accessed by SEM analysis and it was possible to observe that in glucose-grown cells *RLM1* mutants displayed biofilm-like structure more evident than WT and complemented strains, but, no phenotypic differences between the WT and mutant cells were observed when grown in biofilm (Figure 17). The structure of biofilm by SEM was only observed in WT, SCRLM1M4B and SCRLM1K2B, since the results even with some variations seems to have the same trend, which complemented strains (SCRLM1K2A and SCRLM1K2B) presents similar behavior within them and as WT, and the mutants strains (SCRLM1M4A and SCRLM1M4B) also presents similar behaviors between them. This result is in accordance with the previous work of Delgado-Silva (2014).

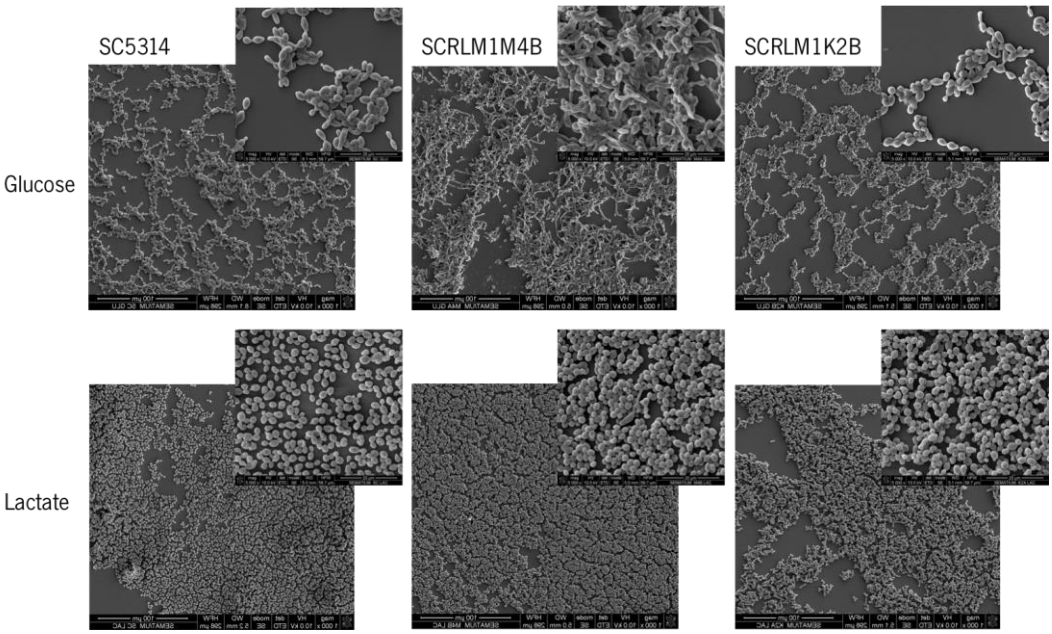

Figure 17. In vitro ability of wild-type, mutant, and complemented strains to adhere to polystyrene surface. SEM analysis of adhered cells of wild-type SC5314 strain (*RLM1/RLM1*), the homozygous *RLM1* mutant strains SCRLM1M4A and SCRLM1M4B (*Δrlm1/Δrlm1*), and the complemented strains SCRLM1K2A and SCRLM1K2B (Δrlm1/Δrlm1+ RLM1) after 48 hours of incubation.

However, when *C. albicans* cells were grown on lactate more adherent cells were observed and the structure of those cells was much similar to a biofilm structure than the glucose-grown cells, which displayed a monolayer of cells. In lactate-grown cells no phenotypic differences was observed between the mutant strains and the WT, as observed for glucose-grown cells.

Delgado-Silva et. al (2014) observed that absence of *RLM1* significantly activated genes involved in cell adhesion and biofilm formation (Delgado-Silva et al. 2014). Blankenship et al. (2010) had already observed novel functions in *C. albicans* conserved protein kinases (PKs) compared to their orthologs in *S. cerevisiae* and *Schizosaccharomyces pombe* when studying cell wall remodeling, which included functions related to filamentation and biofilm formation (Blankenship et al. 2010). Nobile et al. (2009) described Zap1/Csr1 also as a negative regulator of biofilm formation and in a previous study Nobile and Mitchell (2006) identified Bcr1 as a transcription factor that governs biofilm formation in an in vitro catheter model (Nobile and Mitchell 2006; Nobile et al. 2009). Delgado-Silva et. al (2014), demonstrated that *RLM1* behaved as a negative regulator of biofilm in an in vitro polysteryne biofilm model, and the *RLM1* mutant strain presented overexpression of *ALS1*, *ALS3*, *HWP1*, *RBT1* and *ECE1*, which includes the same Bcr1 targets (*ALS1*, *ECE1*, and *HWP1*), suggesting that *BCR1* activates genes directly involved in biofilm formation while *RLM1* regulates negatively the same set of genes (Delgado-Silva et al.

2014). Additionally, lactate as being the main carbon source for *C. albicans* acts as synergetic with the lack of functional *RLM1*.

3.5. Secreted Aspartyl Proteinases production (SAPs)

The expression of secreted aspartic proteinase (SAP) genes is regulated in response to carbon source and nitrogen available (Hube et al. 1994). For example, *SAP2* is expressed at high levels during growth on glycerol, at medium levels on glucose or galactose, and at low levels when *C. albicans* cells are grown on ethanol (Hube et al. 1994).

In this study, the SAPs production by *C. albicans* grown on glucose and or on lactate was evaluated with two different methods. One of them was assessed using a "classical" YCB-BSA agar plate and the other method used YNB-BSA agar plate supplemented with glucose or lactose, since the interest of this study was to evaluate the impact of an exclusive carbon source, by eliminating the influence of YCB.

The results depicted in Figure 18 demonstrated that only glucose-grown cells were able to produce SAPs in both methods, however the SAPs were also produced on YCB-BSA agar plate by lactate-grown cells. No differences were founded in the length of the halos in all strains for each condition, which suggests that *RLM1* is not involved in the SAPs production. However it is possible to note that the halos of glucose-grown cells are slightly higher than those of lactate-grown cells.

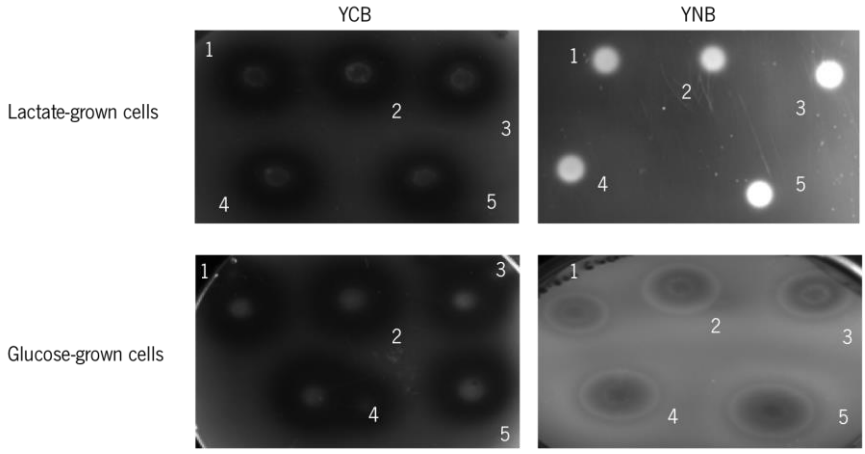

Figure 18. Secreted aspartyl Proteinases production of *C. albicans* in different carbon sources. 1-SC531; 2-SCRLM1M4A; 3-SCRLM1K2A; 4-SCRLM1M4B and 5-SCRLM1K2A.

As observed in the Figure 18, in fact, lactate-grown cells were able to produced SAPs, however this production might be masked by the influence of YCB. Up until now, no information about SAPs production was reported for lactate-grown cells.

Regarding the *RLM1* mutant, as reported before, no difference was observed in the mutant compared with WT. Delgado-Silva et. al (2014) demonstrated that lack of *RLM1* leads to an up-regulation of a secreted protease (*SAP6*), but this up-regulation does not induce an higher halo of degradation. *RLM1* mutant also up-regulated genes involved in the uptake of amino acids and oligopeptides *AGP2* and *OPT6*, respectively. These, together with the action of *SAP6* contribute to the intake of small peptides and aminoacids. The up-regulation of *PUT2* and *GCV2*, whose products are involved in amino acid degradation, may suggest the utilization of alternative carbon or energy sources by *C. albicans* mutant cells (Delgado-Silva et al. 2014), but still no information was reported about non-fermentable carbon source, such as lactate.

3.6. Influence of carbon source *RML1*-responsive genes

In this study, gene expression was analyzed in cells grown on glucose and or on lactate, rather than YPD or under any cell wall stress condition such as in previous works (Delgado-Silva et al. 2014; Bruno et al. 2006). Data was analyzed by using a ΔΔCt method and presented as the fold difference in expression relative of the target genes to wild-type (WT) gene expression, in *C. albicans* cells grown on glucose and or lactate (Figure19). These genes were chosen according to our previous work and are involved in several different biological processes from cell wall organization (*ALS3*, *HWP1*) to metabolic and stress response (*GPD1*, *AGP2*, *GCV2*, *SOU1*, *PUT2* and *CIT1*) Delgado-Silva et. al (2014). This analysis presented is for mutant strain SCRLM1M4B and for complemented strain SCRLM1K2B, since the all data obtained with these strains are representative of all the other strains.

Comparing the ratio of expression of the tested genes in cells grown on glucose or lactate (Figure 19A) it is clear that the majority of the genes follow a similar pattern, except for *SOU1* and *ALS3.* This indicates in the mutant behaves similarly in both carbon source in respect to these genes. Sou1p has ben involved in utilization of L-sorbose while Als3p is a protein is a cell wall adhesion. Regarding the other genes the ratio of expression in the mutant was close to 1, except AGP2, CIT1 and PUT2 that were close to or higher than 2.

Cit1p has been described as a citrate synthase, the enzyme responsible for the first step of the TCA cycle, so the absence of a functional *RLM1* affect this pathway differently. Agp2p is an amino acid permease, suggesting the utilization of alternative carbon/energy sources in the absence of *RLM1.* Put2p has been reported to be involved in amino acid degradation, which may

suggest once more the utilization of alternative carbon or energy sources by *C. albicans* mutant cells.

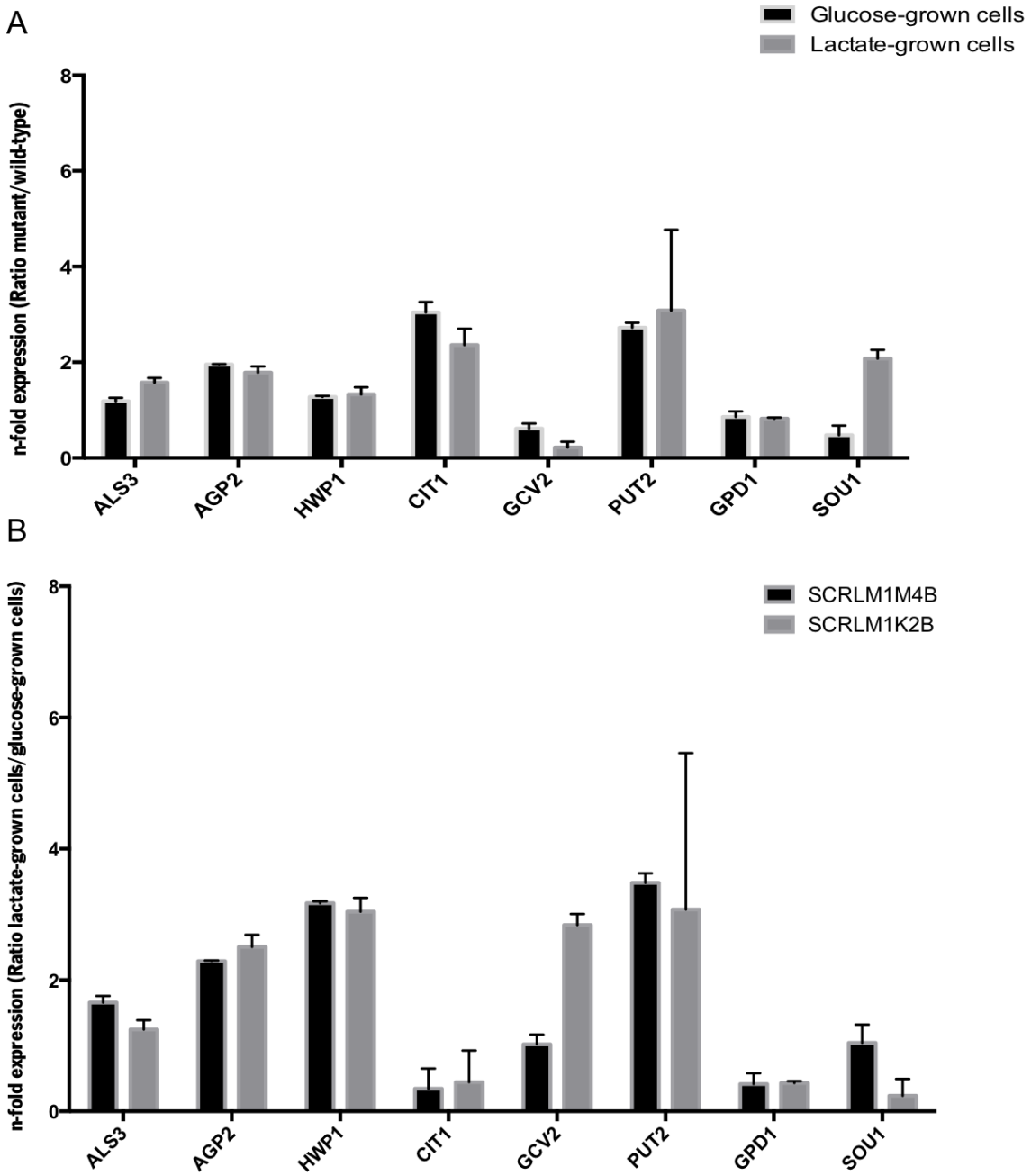

Figure 19. Expression of genes of *C. albicans* strains grown on different carbon sources. The values are present in relative expression ration mutant/wild-type (A) and relative expression ration lactate-grown cells/glucose grown cells.

Relatively to the effect of carbon source, namely lactate, in the expression of these genes in the mutant and in the complemented strain (Figure 19B), it is possible observe that in lactate-grown cells the expression of *ALS3*, *AGP2*, *HWP1*, *GCV2* and *PUT2* are higher than in glucose-grown cells (expression higher than 1.0).

In a certain way, these results confirm some previous results presented in this study, as we observed the high ability of adhesion displayed by lactate-grown cells, which may correlate with a higher expression of adhesion related genes, *ALS3* and *HWP1,* in those *C. albicans* cells.

As reported before, *AGP2* and *PUT2* as well as *GCV2* are much influenced when *Candia* cells are grown on lactate, since these genes are involved in the metabolism of alternatives carbon source such as amino acids up-taking.

Another interesting date observed in this analysis is that *GPD1* was barely influenced by lactate as primary carbon source. *GPD1* was one of the genes that Delgado-Silva et. al (2014) observed a down-regulation in the mutant. The product of this gene is the glycerol-3-phosphate dehydrogenase, involved in the glycerol biosynthesis pathway, and in this present work, this gene was also down regulated in the mutant when *C. albicans* cells were grown on lactate as well as when cells were grown on lactate. However, the relative expression of *GPD1* was lower in cell grown on lactate than on glucose-grown cells and no differences were founded within the strains, which may be due to its specific function related with carbohydrates metabolism. Since this gene was founded down-regulated upon the *RLM1* deletion, according to Delgado-Silva et. al (2014) work, suggest *RLM1* mutant might use other carbon source than lactate or glucose.

Other genes that could be involved in the interconnection of the pathways required to metabolize non-fermentable carbon sources, i.e. involved in the gluconeogenesis, the glyoxylate cycle, and beta-oxidation, are *CIT1*, coding for citrate synthase and *SOU1* a sorbose redutase, all up-regulated in the mutant (Delgado-Silva 2012; Delgado-Silva et al. 2014). *CIT1* was up-regulated in the mutant, either on glucose and lactate grown cells, suggesting that its expression was not influenced carbon source. However, comparing the expression ration on lactate/glucose, it is clear that its expression is heigher in glucose grown cells, either in the mutant and complemented strain. Regarding *SOU1*, this gene behaved differently in the mutant when cells were grown on the two carbon sources. In cells grown on lactate SOU1 is up-regulated in the mutant while in cells grown on glucose is down-regulated. This result suggests that sorbitol may have an important role in the metabolization of lactate.

3.7. Host-pathogen interaction: Participation of *RLM1* in Macrophage-*Candida albicans* interaction under the influence of different carbon source

To determine *RLM1* impact in the host-pathogen interaction we investigated the ability of the *Δrlm1/Δrlm1* mutant strain to interact with macrophages murine cell line J774, taking into account the influence of different carbon source, glucose and lactate. To assess this host-pathogen interaction, the macrophages were incubated with live *C. albicans* cells in a MOI of 5:1 for 1h. The uptake of live fungal cells by macrophages was measure by counting CFUs (colony forming units) and the results presented in percentage of Yeast Killing (Figure 20). Furthermore cytokines were also measured in order to understand the immune response in the context of this study.

Results indicate that lactate-grown cells were less efficiently killed by macrophages (around 20%) than glucose-grown cells (around 50%) (Figure 20).

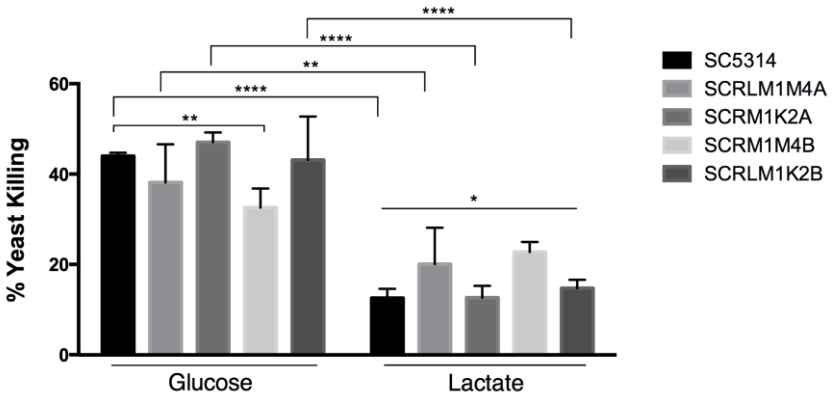

Figure 20. Yeast Killing. Killing of *C. albicans* SC5314, SCRLM1M4A, SRLM1M4B, SCRLM1K2A and SCRLM1K2B cells grown on glucose, lactate by macrophages. Fungal cells and macrophages were incubated for 1h at 5:1 *C. albicans*/macrophage ratio. Values represent means ± SD for 2 independent experiments. Results are expressed as the percentage of yeast killing. The *C. albicans*/macrophage ratio was 5:1.

Considering *RLM1* influence in the interaction between *C. albicans* cells and macrophages it seems that *Δrlm1/Δrlm1* mutant strains are less susceptible to macrophage killing when grown on glucose, in comparison with the WT and complemented strains. The exact opposite was observed when cells were grown on lactate.

To determine the effect of the disruption of *RLM1* on the immune response elicited by *C. albicans* grown on glucose or on lactate, the pro-inflammatory cytokine TNF-α and the anti-inflammatory IL-10 were measured in after 1h incubation with murine macrophage-like cell line J774A after challenged with *C. albicans* cells from WT, mutant or complemented strains (Figure

21.A and B). In Figure 21A, the results showed that in cells grown on glucose TNF-α levels produced by macrophages were significantly lower in response to *Δrlm1/Δrlm1* mutants cells in comparison with WT (P <0.0001). On the other hand, the complemented strains, although inducing a higher amount TNF-α than the mutant strain, present even a little bit higher levels than WT. Regarding the impact of the carbon source in this host-pathogen interaction it was clear that lactate-grown cells stimulated increased TNF-α production than glucose-grown cells. An interesting data can be also observed in TNF-α production by lactate-grown cells lacking of *RLM1*, the trend observed in glucose-grown cells for *Δrlm1/Δrlm1* mutants is reverted when grown on lactate (Figure 21A).

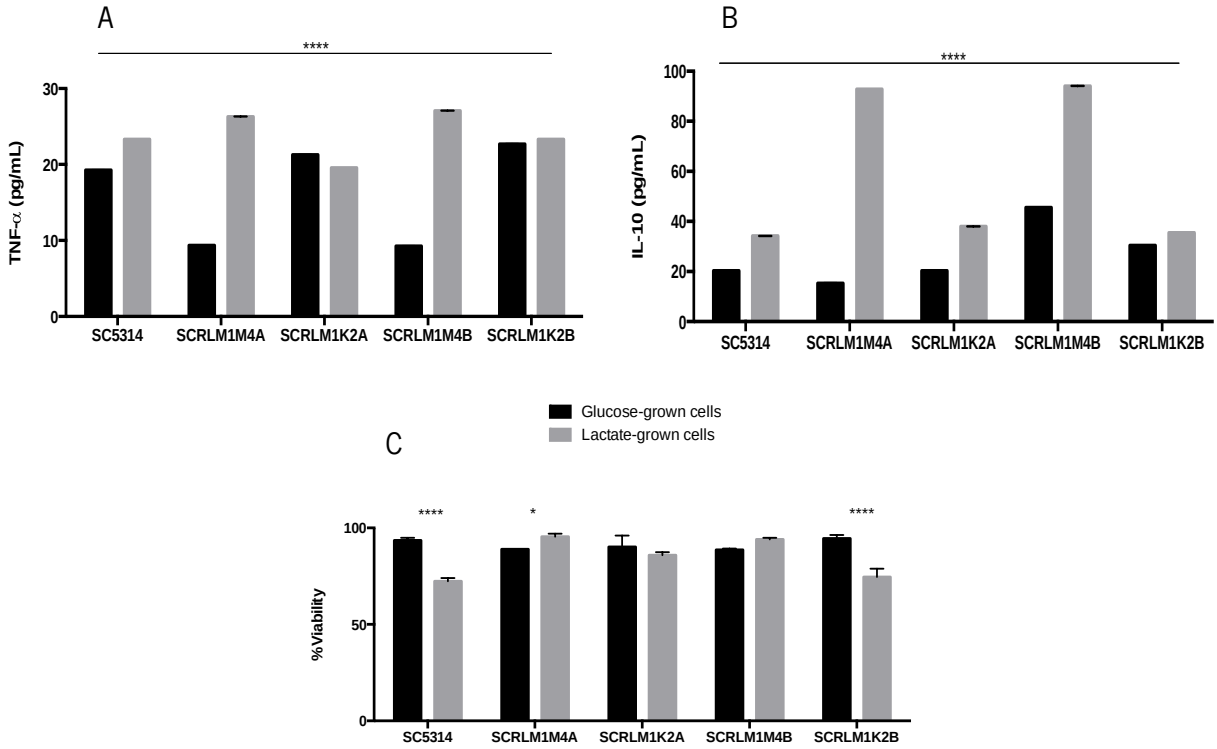

Figure 21. Host vaibility and immune response to *Candida* cells grown on glucose or on lactate.

Then the anti-inflammatory IL-10 was measured in similar conditions as described before for TNF-α (Figure 21B). The results for IL-10 production was in general similar to those observed for TNF-α, with exception for the mutants and complemented strains B. The same trend was observed in lactate-grown cells, which presented higher production of IL-10 than glucose-grown cells. Also, the immune response to the mutant strains grown on lactate was reverted too, as observed for the TNF-α production.

In order to quantify the cell damage caused by the WT, *Δrlm1/Δrlm1* mutants and complemented *C. albicans* strains, we determined the LDH released from macrophages after 1h of co-incubation. As illustrated in Figure 21C, the cell damage caused by Δrlm1/Δrlm1 mutant was a little higher in comparison with the WT and complemented strains, in cells grown on glucose, however in lactate-grown cells these effect was the opposite, which is possible to observed that *Δrlm1/Δrlm1* mutants caused less cell damage than WT and complemented strains. These results indicate that the deletion of *RLM1* has a significant impact in the immune response as well as the carbon source.

The host response to infection by *C. albicans* is associated with the release of different cytokines pro- and anti-inflammatory, such as TNF-α and IL-10, respectively, and the balance between them determines the fade of *C. albicans* (Mencacci et al. 1998). The comparative analysis of the interaction of the WT, *Δrlm1/Δrlm1* mutant and complemented *C. albicans* strains with J774 macrophages cell line showed that at 1h of co-incubation, in glucose-grown cells, the levels of TNF-α were much higher, in response to WT and complemented strains in comparison with *Δrlm1/Δrlm1* cells. These observations are in agreement with previous work of Delgado-Silva et. al (2014) that sowed by histopathology results that kidney sections revealing heavy infiltration of leucocytes around *C. albicans* cells, in mice infected with WT or complemented strains and few inflammatory cells around the *Δrlm1/Δrlm1* mutant cells (Delgado-Silva et.al 2014).

Previous works (Ene et al. 2012b; Ene et al. 2012a) as well as this present study demonstrated that *C. albicans* grown on alternative carbon sources induced fungal cell wall remodeling and modulation of the cell wall proteome and secretome (Ene et al. 2012b; Ene et al. 2012a). This fact already described will affect and regulate important virulence attributes, such as stress and drug resistance, adherence, biofilm formation, and infection outcome.

IL-10 represents one of the key immune cytokines for the host defense against *C. albicans* infection. Lactate-grown cells display a significant reduction in the amount of mannan in the cell wall as well as in the organization of the mannan fibrils (Ene et al. 2012a). The amount of β-glucan is also decreased in the lactate-grown cell wall, but its recognition might be masked by the altered architecture of the mannan fibrils and the different proteins attached to it (Ene et al. 2012a; Ene et al. 2012b). IL-10 drives a Th2 response and it as also play an important role in the host defense against disseminated candidiasis (Romani 2004). Increased IL-10 production, modulated through different TLRs and dectin-1, shifts the balance toward anti-inflammatory cytokine responses

(Gringhuis et al. 2007). On the other hand, the upregulation of IL-10 also exacerbates *Candida* infection in mice (Tonnetti et al. 1995). IL-10-deficient mice are more resistant to *Candida* infection, due to an up-regulated Th1 antifungal response (Del Sero et al. 1999). Hence, increased IL-10 production might predispose the host to *Candida* infection. The importance of *RLM1* in the interaction between macrophages and *C. albicans* cells seems to be different behaviors when cells are grown on glucose or on lactate. *RLM1* mutant strains when grown on lactate as well as on glucose seem to behave at the same level in comparison with WT strain, observing a switch of cytokines production when carbon source changes from glucose to lactate. This data lies on the previous results here presented that the cell wall suffers remodeling when grown lactate as well as the fact that *RLM1* mutant strain present different organization in its cell wall "per se" concluded by Delgado-Silva et. al (2014).

These observations confirmed that *RLM1* as well as carbon source impacts in the recognition and phagocytosis of *C. albicans* cells by macrophages, further reinforcing the idea that differential nutrient availability in host niches significantly affects host-fungus interactions. The mechanist of the *RLM1* together with effect of different carbon source needs to be further clarified.

Concluding remarks

4. Concluding remarks

Candida albicans, as an opportunistic pathogen, is able to adapt its growth to a range of environmental changes, by modulation of expression of many genes in a coordinated manner. Metabolic adaptation impacts upon *C. albicans* pathogenicity at multiple levels: by promoting nutrient assimilation, cell wall remodeling, stress resistance, and the expression of virulence factors, and also by influencing immune surveillance (Figure 22) (Brown et al. 2014a).

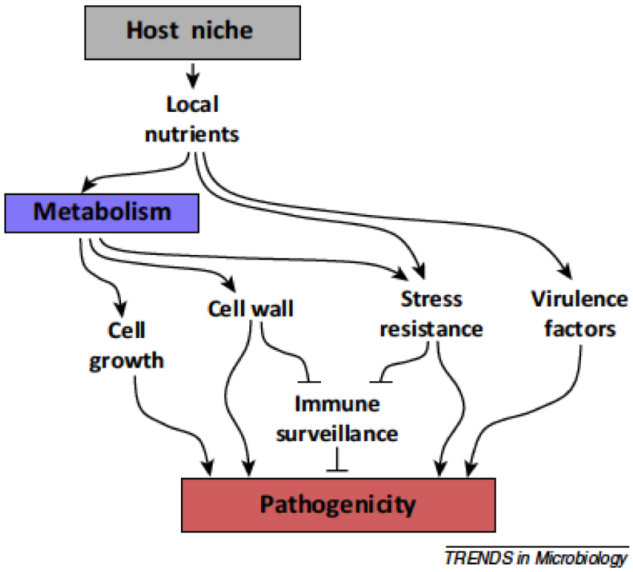

Figure 22. Nutrient adaptation affects *Candida albicans* pathogenicity at multiple levels. Nutrient adaptation contributes directly to pathogenicity by supporting fungal growth. Nutrient adaptation also promotes pathogenicity indirectly through cell wall remodelling, by enhancing stress resistance, by modulating the expression of key virulence factors, and by affecting the efficacy of immune surveillance by innate imune cells. Adapted from (Brown et al. 2014a).

The cell wall, as the most external cellular component, plays a crucial role in the interaction with host cells mediating processes that are essential during infection, hence the importance of carrying out studies about cell wall molecules that participate in the virulence of this yeast (Delgado-Silva et al. 2014). Cell wall has been characterized in the model organism *S. cerevisiae*, with which *C. albicans* shares many orthologous proteins, including *RLM1* (Delgado-Silva et al. 2014). *RLM1* belongs to the type II MADS-box proteins family characterized by a genetically variable C-terminus due to the presence of a repetitive region of different sizes (Sampaio et al. 2009; Delgado-Silva et al. 2014). *C. albicans Δrlm1/Δrlm1* mutant, the complemented and WT strains were exposed to several cell wall stress agents under the influence of different carbon sources. In this study, *C. albicans Δrlm1/Δrlm1* mutant displayed phenotypes associated to cell wall deficiency such as hypersensitivity to CR and CFG either on glucose and lactate grown cells. These results and the homology with MADS-box transcription factors as well as the impact of

different carbon source suggest an involvement in a regulatory role of cell wall remodeling. This regulatory role was further evidenced by the nuclear localization of *RLM1-GFP* fusion protein observed in Delgado-Silva et. al (2014) work. In this study, different sensitivities to SDS and Caffeine was observed in lactate-grown cells in comparison with glucose-grown cells, indicating that lactate-grown cells presents higher sensitivity to those cell wall stress. In accordance to Delgado-Silva et. al work, gene expression in the mutant lacking *RLM1* significantly activated genes involved in cell adhesion like *ECE1*, *ALS1*, *ALS3*, *HWP1* and *RBT1*, and decreased transcription of genes involved in the catabolism of carbohydrates, *DAK2*, *GLK4*, *NHT1* and *TPS1* (Delgado-Silva et al. 2014). It has been described also that lactate-grown cells have higher capacity of adhesion than glucose-grown cells (Ene and Brown 2014). The increased transcription of genes involved in cell adhesion as well as the impact of carbon source was confirmed by adhesion and biofilm assays, suggesting that *RLM1* acts as a negative biofilm regulator even grown on lactate.

In addition, decreased production of TNF-α and IL-10, and lower cellular toxicity was observed in the absence of a functional *RLM1*, suggesting that *RLM1* has a significant impact on *C. albicans* virulence. However these phenotypes were reverted when cells were grown on lactate, which indicates that nutrients availability confers alterations in both interlayers resulting in different outcomes in host-pathogen interaction.

Finally, qRT-PCR analysis showed that the expression of *ALS3*, *HWP1*, *AGP2*, *PUT2*, *GCV2*, *CIT1*, *SOU1* and *GPD1*, which are genes involved in distinct biological processes such as cell adhesion and metabolism of alternative carbon sources was, in general, higher in the *Δrlm1/Δrlm1* mutant in relation to the WT strain, however this trend was observed more when *Candida* cells were grown on lactate, even though the these results need to be further repeated in order to observe a clearly a trend.

Overall, these results showed that the transcription factor *RLM1* is involved in the stability of the cell wall, in the interaction with the host, and being important for the virulence of *C. albicans.* These observations also showed that *C. albicans* mutant appears to rearrange the metabolic pathways so that glucose or lactate is deviated from utilization as energy source, being more available for use as cell wall building blocks, as well as increase cell wall protein involved in adhesion. As a consequence the association between β-glucan, mannoproteins, and chitin would certainly change (Delgado-Silva et al. 2014).

As discussed above, we concluded that *RLM1* is important for the cell wall biogenesis and adaptation to different carbon source might change perceptions how *Candida*–host interacts.

Perspectives for future work

5. Perspectives for future work

The studies performed in this work allowed to achieve the main objectives proposed for this thesis. In spite of the advances obtained in this work, regarding molecular and functional characterization *RLM1* gene, new questions have emerged that should be covered in the future.

Some of future has already been done such the characterization of possible alterations in organization and cell wall contents of *RLM1* mutant under lactate growth condition, hence *RLM1* mutant displayed "per se" alterations in its cell wall and it is wide described that different carbon source has impact in modulation of cell wall (Delgado-Silva et al. 2014; Ene et al. 2012a; Ene et al. 2012b).

Proteomic studies of *RLM1* mutant is also an interesting field to explore and we believe that cell wall proteins might be different in lactate-grown cells than glucose-grown cells under the influence of lacking od functional *RLM1*.

Since *RLM1* is a transcriptional factor, some studies of transcriptomic is also need to understand what happens downstream of *RLM1*-dependent pathways, such as CWI under the influence of lactate as primary carbon source. In this way the importance of *RLM1* as a virulence factor can be further demonstrated which will open the perspective to new strategies of antifungal therapies targeting kinases of the CWI pathway and, in particular, the transcription factor *RLM1*.

Since *RLM1* mutant have up-regulated genes for use alternative carbon source it is also interesting to explore other carbon sources than glucose or lactate.

In order to better understand the impact of *RLM1* in host-pathogen interaction under the influence of different carbon source is important to preformed additional studies including other immune cells as well as studies regarding immune-response pathway, such as mTOR pathway or the glyoxylate cycle.

Finally, it is also important to exploit this study using clinical strains, such as 124a (Sampaio et al. 2010).

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6. References

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