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Candida Albicans

Edited by Doblin Sandai

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Meet the editor

Associate professor Dr. Doblin Sandai is a molecular medical mycologist. He graduated from the Institute of Medical Sciences, University of Aberdeen, Scotland, UK. Currently, he is working as a university lecturer at the Infectomics Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia Bertam, Penang, Malaysia. His research is centered on virulence factors, proteins, and gene regulation, as well as developing alternative inhibitors from natural products to combat infections and resistance of the pathogenic yeast *Candida albicans*. Associate professor Sandai is involved in teaching and supervising postgraduate students. He has published over 40 articles, including books and peer-reviewed journals related to carbon source attribution to the pathogenicity of *C. albicans*. He is an international member of the American Society of Microbiology and the International Society of Human and Animal Mycology.

Contents

Preface

Candida albicans and other medically relevant *Candida* species are mainly commensal yeasts commonly found on mucosal surfaces and in the gastrointestinal and genitourinary tracts of humans. These yeasts are opportunistic pathogens that are mostly benign, but can be infectious when the host immune system becomes impaired or an environmental niche becomes available. The infections caused by *Candida* species are generally known as candidiasis, which can be further classified into superficial infections such as oral thrush, vaginal candidiasis, and invasive candidiasis. Invasive candidiasis is one of the most common nosocomial invasive infections with a high mortality rate, even with first-line antifungal treatments. This book will mainly focus on *C. albicans* and is divided into four sections, namely emerging fungal diseases, adaptation and resistance, interaction of cell hosts and other microbes, and new therapeutics and management.

Section 1 starts with an overview of the emerging and emerged pathogenic *Candida* species. These chapters will offer a historic perspective of *C. albicans* and other non-albicans (NAC) that cause serious disease and will review the current knowledge of emerging NAC pathogens with useful graphics and current references. Also, the articles consulted great variations of *Candida* species were tested and in MIC concentration from the development of new natural products, displayed the best performing plants that are effective in control of oral and systemic candidiasis, cheap and accessible for the population.

Section 2 highlights the unique metabolic adaptation of *C. albicans* that relates to the successful assimilation of available carbon sources during infections and resistance. Also, it has been proven that host carbon sources, blood serum, high temperature, acidic environments, and morphogenesis have a direct impact on mannoprotein content. Therefore, these changes might be involved in the adherence, drug resistance, and virulence of *C. albicans*. The proteomics data obtained were performed via comparative analysis of the proteins found in cell walls, whose primary location and function have been reported in different cellular compartments.

Section 3 covers important issues regarding the common role of phagocytes in antifungal immunity, defects in other immune cells, and immunologic pathways that give rise to susceptibility to infection with *Candida* and other fungi. Cell-mediated immunity is essential for antifungal immunity. This is evidenced by the predisposition to severe fungal infection in infants with severe combined immunodeficiency, a life-threatening condition manifested by low, absent, or severely dysfunctional T cells. Also, the mechanism of interaction between *C. albicans* and diverse microorganisms, including bacteria and yeast-like fungi from the genus *Candida*, is discussed in these chapters.

Section 4 is a special section on the therapeutics and management of fungal infections and new treatment strategies that involve the use of nanotechnology to overcome challenges as-

sociated with antifungal therapy. This review focuses on various means that have been employed to effectively counter problems supplementary to *C. albicans* by using nanotechnology.

This book is an updated overview of collected data on *C. albicans* research and review information. Clinicians and researchers should promptly manage pre- and post-candidiasis before proceeding to more sophisticated intensive investigation. Lastly, proper infection control principles should be kept in mind at all times to avoid invasion by this opportunistic pathogenic yeast.

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Section 1

Emerging Fungal Diseases

Chapter 1

Emerging Pathogens of the Candida Species

Bo Yang and Reeta Rao

Additional information is available at the end of the chapter

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Abstract

In recent years, opportunistic and nosocomial fungal pathogens have been dominated by yeasts of the genus *Candida*. Most of the research has focused on *Candida albicans* since it is the most prominent etiological agent. There are numerous publications that describe the biology, virulence factors, morphology, immunity, genomics, diseases, and laboratory aspects of *Candida albicans*. In this chapter we offer a historic perspective of *C. albicans* and focus on other non-albicans candida (NAC) that cause serious disease. We review the current knowledge of emerging NAC pathogens with useful graphics and current references. This chapter is laid out as an overview and is geared for students seeking basic information and may be superficial for an infectious diseases clinician.

Keywords: *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. guilliermondii*, *C. auris* and *C. krusei*, non-albicans candida (NAC), fungal pathogen

1. Introduction

By the end of the twentieth century, hospital-acquired fungal infections were on the rise. Infections by yeasts of the genus Candida have become one of the most common causes of bloodstream infections [1]. The increase in fungal infections is typically attributed to the longer survival of immuno-compromised individuals as well as the increase in the number of people in long term health care facilities undergoing, immunosuppressive therapy, long-term catheterization, broad-spectrum antibiotic use among others. This alarming increase in nosocomial fungal infections has alerted clinicians and scientist that yeasts, previously thought innocuous and relegated to plant pathology or industrial use were capable of causing serious illness.

While infections caused by Candida species are typically superficial and restricted to the urogenital or mucosal oral cavities, they are also capable of entering the bloodstream leading to deep-tissue infections [2].

The predominant yeasts in bloodstream infection remain restricted to the genus Candida [3] most of which, belong to the CTG clade, where the CTG codon is translated as Serine rather than Leucine [4]. Although the recent rise in the number of these infections [5] is mainly associated to *C. albicans*, non-albicans candida (or NAC) related diseases are also increasingly reported in different parts of the world [6]. The relative rates of infection among all Candida infections are shown in **Figure 1**. There are at least a dozen *Candida* species that can be pathogenic for humans, but more than 90% of reported invasive infections are associated with *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis* [7] .

The definition of a new or "emerging" pathogen is subjective at best. For example, how many independent isolations are required before an emerging pathogen is established as an infectious agent? Indications of emerging infections typically consist of case reports. The incidence of yeast infections is likely under-reported because it is dependent on clinical diagnosis and their desire to investigate, then confirm the novelty of the etiological agent, write a compelling report, and to withstand the critique and rigor of publishing the report. A close partnership between a research scientist and a clinical physician is critical for the most rigorous reports.

Furthermore, a single report describing several cases of infection by a novel microbe does not necessarily indicate that a new infection has emerged. Since these temporally separated clinical samples must be confirmed as being independent and not affected by laboratory practices, personnel and/or environmental factors [8]. For example, recent retrospective studies of blood stream infection caused by the genus *Candida*, reported *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. guilliermondii*, and *C. krusei*. This list closely resembles the list of known pathogenic Candida species in 1963 [3] suggesting that the emergence of other yeasts

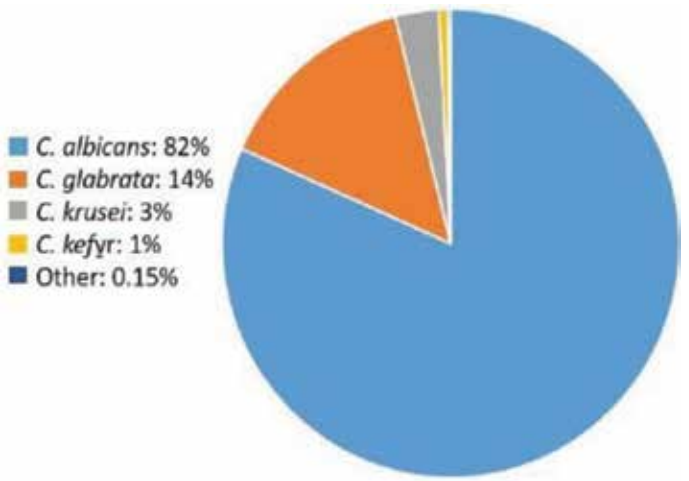

Figure 1. Relative rates of infection with Candida species.

as potential bloodstream isolates is a reflection of the changes in medical and laboratory practices since the early 1960s. Another important variable is that the advances in genomic tools coupled with the ability of contemporary blood culture systems and procedures to support the growth of the unusual yeast isolates have greatly accelerated the frequency of isolation and identification of novel (non-*C. albicans*) isolates as important pathogen.

For the purposes of this chapter we limit the definition of emerging pathogens to those that have recently appeared within a population or those whose incidence or geographic range is rapidly increasing or threatens to increase in the near future or those caused by previously undetected or unknown infectious agents.

2. Established pathogens of the Candida species

2.1. *Candida albicans*

The first description of a yeast infection was of thrush, by Hippocrates in the fifth century B.C. [9]. Since its first microscopic detection in thrush swabs by Langenbeck, subsequently Berg and Gruby [9] in 1839, *Candida albicans* has been confirmed to be the primary etiologic agent of thrush and cause of many other forms of mucosal disease. In fact, *C. albicans* is most frequently isolated species from human yeast infections [3]. Here we touch upon some of key features that make *C. albicans* a successful pathogen. The various morphological forms of *C. albicans* (**Figure 2**) have been associated with the shift from commensal or pathogenic states. The change from yeast to hyphae is thought to help cell adhesion and facilitate tissue infection, macrophage evasion and biofilm development [10].

C. albicans is a diploid polymorphic yeast (**Figure 2**) of human mucosal surfaces, a fungus that commonly found in the gastrointestinal (GI), respiratory, and urogenital tracts. It is generally commensal but able to turn into an opportunistic pathogen in immunocompromised or immuno-deficient individuals. It is the major species causing invasive candidiasis (46.3%), followed by *C. glabrata* (24.4%) and *C. parapsilosis* (8.1%) [11]. Systemic infections of *C. albicans* often cause death with a mortality rate of ~40% [12]. One reason leading to over growth of *C. albicans* is its ability to respond to a myriad of environmental imbalances such as changes in nutrition, temperature and pH [13]. Other important factors that increase the risk of *C. albicans* infection are prolonged treatment with broad spectrum antibiotics, surgical procedures, various diseases such as diabetes, trauma, and other genetic disease or congenital malformation. [2].

Candida albicans belongs to the CTG clade of fungal species and translates canonical leucine codons, CTG, to serine [14]. It prefers to use glucose as the carbon and has multiple approaches to transport and metabolize glucose. Nutritional changes alter virulence of *C. albicans* both during systemic infection as well as mucosal vaginitis, suggesting that alternative carbon sources within host environments are important during *C. albicans* infections [15].

Post-transcriptional mechanisms underlying this transition include mRNA stability, alternative transcript localization, and translation and influence *C. albicans* virulence processes. Below we highlight some key pathways but for details refer to book Candida and Candidiasis [16].

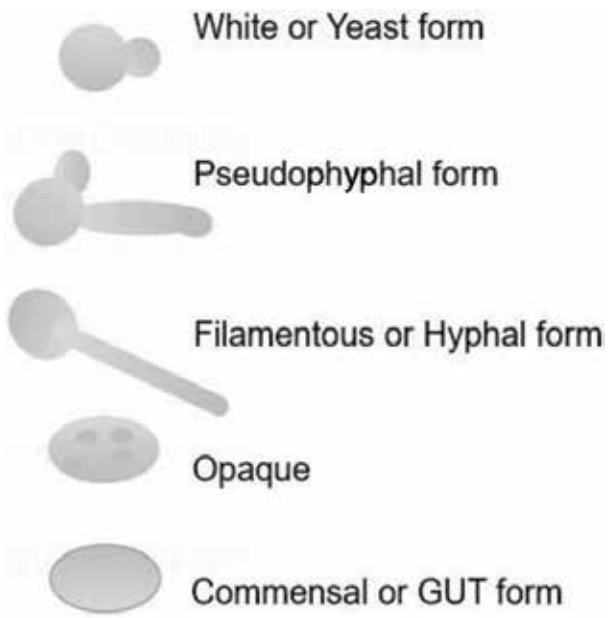

Figure 2. *Candida albicans* polymorphology.

At the transcriptional level, *C. albicans* fibrin Sac6 modulates morphogenesis and oxidative stress responses [17]. As mentioned above, metabolic adaptation a key virulence determinant is involved in the susceptibility of *C. albicans* to antifungal drugs as well as stress resistance and innate immune responses [18]. *C. albicans* typically infects epithelial cells through two specific mechanisms, active penetration and endocytosis. Before the infection, *C. albicans* transfer between commensal and invasive states through distinct genetic pathways to regulate the expression of hypha-specific and/or phase-specific genes. And these genes express proteins to regulate directly or indirectly to the pathogenesis and virulence of *C. albicans* [19]. The gastro-intestinally induced transition (GUT) highlights how these pathways are used [20]. Superficial candida infections require the interaction between fungal cell surface proteins or pathogen associated molecular patters (or PAMPs) with host innate immune cells system or pathogen recognition receptors (or PRRs) (**Figure 3**). For example, cell-wall β-glucans can stimulate monocyte reprogramming as one of the main immunological responses in hosts [2].

When interacting with macrophages, the SPS system is stimulated in the nutrient poor host environment and is critical for resistance of *C. albicans* to macrophages. It consists of three components; an amino acid transporter, SSY1, a membrane associated protein PTR3 and a chymotrypsin-like serine endoprotease, SSY5. Under conditions of carbon deprivation, signaling through the Stp2 transcription factor triggers the use of amino acids as carbon source which helps neutralize the acidic environment of the phagosomes [21]. *C. albicans* employs several mechanisms to evade immune detection (**Figure 3**, [22, 23]).

C. albicans has significant phenotypic and genetic diversity. It contains a diploid genome of 14.4 megabases arranged within eight chromosomes [24]. The heterozygosity and heterozygous

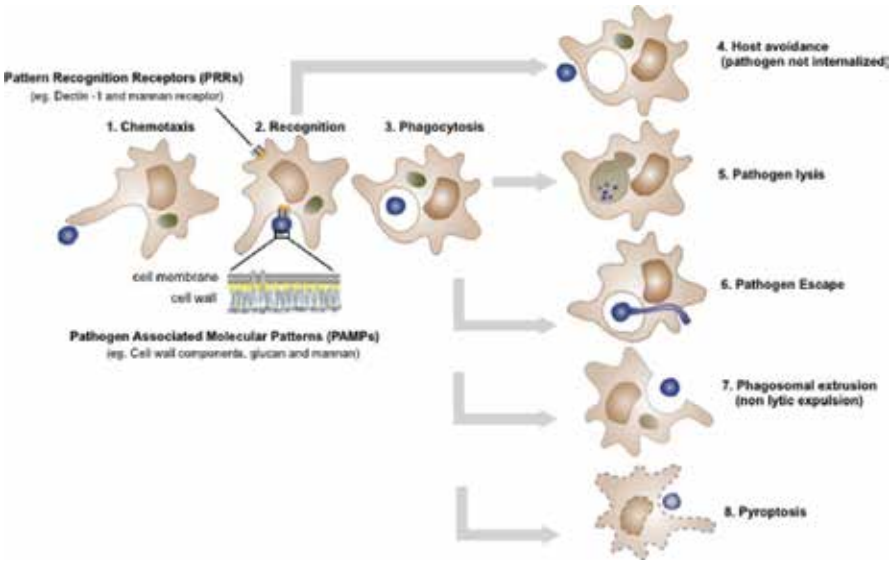

Figure 3. Host-Pathogen interactions. 1. Immune cells chemotax towards the pathogen, 2. fungal cell wall components pathogen associated molecular patterns (PAMPs) interact with macrophage via Pathogen Recognition Receptors(PRRs) such as Dectin-1 and Mannose Receptor that recognize β-(1,3)-glucan and mannan respectively, 3. Phagocytosis, 4. Host avoidance, 5. Pathogen lysis. 6. Pathogen escape, 7. Phagosomal extrusion where pathogen is expelled without lysis and 8. Pyroptosis.

of genome is thought to be related with *C. albicans* virulence and the genomic instability is crucial in its pathogenesis [2]. Differences specific to strains may contribute on the interaction of *C. albicans* and host [24].

Detection and identification of various yeasts has been a challenge. These yeasts can be distinguished morphologically on CHROMagar or spider media (**Figure 4**). However, genome sequencing is the most reliable method for species identification. In addition, detection of microsatellites also represents a reliable method for molecular typing and genetic analysis of *Candida*. A recent *Candida* distribution study conducted in a hospital, reported a clonal population including 62 identified genotypes among the tested isolates [25]. Beyond that, multilocus sequence typing (MLST) is another valuable method to understand the epidemiology of systemic *Candida* infections [2]. Here the DNA of seven housekeeping genes is sequenced. The results showed that MLST of *C. albicans* isolates are highly reproducible and sensitive. Comparative studies using MLST database of *C. albicans* are available online (http://calbicans.mlst.net/). These studies allowed further stratify the geographic isolation of *C. albicans*. For example, the most common MLST cluster within the *C. albicans* species is defined as clade 1. While, clade 2 is mainly located in the United Kingdom, clade 4 includes isolates from the Middle East and Africa, clade 11 includes isolates from continental Europe, and clades 14 and 17, where various gene clusters are regrouped include isolates from the Pacific region [26].

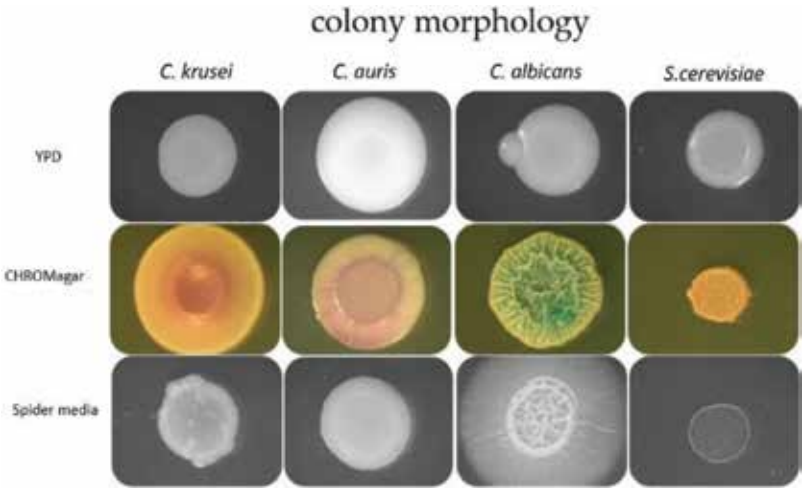

Figure 4. Colony morphology of *Candida krusei*, *Candida auris*, *Candida albicans* vs. *Saccharomyces cerevisiae*.

Pathogen profile: Diploid, belongs to the CTG clade, genome sequence available, antifungal resistance is moderate, molecular laboratory tools available.

2.2. *Candida glabrata*

Candida glabrata is often the second most common cause of candida infections after *C. albicans*. Historically, it has been considered nonpathogenic within the normal flora of healthy individuals without causing serious infection in humans. During the late 1990s, *C. glabrata* genetics was the most advanced among the non-albicans Candida (NAC) species due to its haploid status, its classical codon usage which allows direct usage of *S. cerevisiae* tools), and its high frequency of isolation in hospitals [27]. *C. glabrata* infections can be mucosal or systemic and often occur in immunocompromised persons or people with diabetes [28].

In contrast to most *Candida* species, *C. glabrata* is not dimorphic and exists as small blastoconidia under all environmental conditions both as commensal and pathogenic. In animal models *C. glabrata* is relatively nonpathogenic suggesting that it has few virulence attributes [28]. However, within the host environment, *C. glabrata* spreads rapidly, and is difficult to treat because it is resistant to many azole antifungal agents. Therefore, *C. glabrata* infections have a high mortality rate in compromised hospitalized patients [28].

C. glabrata has haploid genome, in contrast to the diploid genome of *C. albicans* and some NAC species [29]. It is distinguishable from *C. albicans* by its small-subunit rRNA [28]. *C. glabrata* only use glucose and trehalose. Such unique sugar utilization among *Candida* species can be applied to identify yeast to the level of genus and species. Now commercial kits (API 20C, Uni-Yeast-Tek, and YeastIdent) are available to identify *C. glabrata* in mixed cultures [28].

Both *C. glabrata* and *C. albicans* are commensal suggesting that similar host mechanisms such as suppressing expression of pathogenic determinants may be at play to effectively prevent

infection of both microorganisms. However, the relatively low virulence of *C. glabrata* in animal models compared to *C. albicans* suggests genes controlling the virulence of *C. glabrata* may be different from those in *C. albicans* [28].

C. glabrata isolates are often associated with high resistance to the azole class of antifungal agents and less susceptibility to most other antifungal agents including amphotericin B [30]. Several mechanisms of azole resistance of *C. glabrata* have been identified, such as increased P-450-dependent ergosterol synthesis, an energy-dependent efflux pump of fluconazole and possibly via a multidrug resistance-type transporter [28].

Pathogen profile: Haploid, does not belong to the CTG clade, genome sequence available, antifungal resistance is high, several molecular laboratory tools available.

3. Emerging pathogens of the Candida species

3.1. *Candida auris*

Fluconazole-resistant *Candida* has been identified as a serious public health threat (www.cdc.gov). Among these, *Candida auris* has simultaneously and independently emerged on three continents in several countries as a multi drug resistant fungal pathogen with high mortality [31]. Phylogenetic analysis and polymorphism typing indicate that *C. auris* strains are clonal suggesting likely transmission from an environmental source.

Candida auris, as the name suggests, was first isolated from the drainage of the external ear a Japanese patient [31] and 15 Korean patients in 2009 [32]. Identification of it is challenging with standard microbiologic techniques. It frequently exhibits multidrug-resistance. Ever since these initial cases, *C. auris* has become an emerging global health threat causing massive invasive infections and outbreaks in healthcare facilities. Cases have already been identified in India, South Africa, Kuwait, the United Kingdom, Venezuela, Brazil, the United States, Colombia, Pakistan, Spain, Germany, Israel, Norway, and Oman [33].

Phylogenetically, *C. auris* is closer related to *Candida haemulonii* and *Candida ruelliae* [31] while distantly related to other more common pathogens *C. albicans* and *C. glabrata* [34]. Four distinct clades have been identified from geographic separate origins, suggesting almost simultaneous emergence of populations [35]. Susceptibility to antifungal reagents and survival from phagocytosis is largely different among four clades (CDC report).

Risk factors for *C. auris* infection appear to be similar to infections from Candida in general. *C. auris* is reported to grow at temperatures ranging from 35 to 42°C. It forms pink colonies on chromogenic media. *C. auris* does not form pseudohyphae (**Figure 4**) but capable of forming biofilms [36] and adhering to catheter material, although to a lesser degree as compared to *Candida albicans* [37]. Some *C. auris* strains produce phospholipase and proteinase, which may account for the variability in pathogenicity in a murine model [37].

The genomes of several isolates have been sequenced and they appear to parse into four distinct clades by geographic region [35]. Clades were separated by thousands of single-nucleotide

polymorphisms, but within each clade isolates were typically clonal. Various mutations in *ERG11*, the gene encoding for lanosterol 14-alpha-demethylase and induced upon prolonged growth with antifungal drugs were shown to be associated with azole resistance in each geographic clade.

Again, while whole genome sequencing is the most reliable method for species identification, PCR and real-time PCR assays have shown excellent accuracy and have been effective for diagnosis, to rapidly identify *C. auris* [38]. The development of new antifungal medications with activity against *C. auris* will be vital to controlling *C. auris* as therapeutic options are already limited. Also aggressive infection control measures are critical to reducing the spread of *C. auris* [33].

Pathogen profile: Haploid, belongs to the CTG clade, Genome sequence available, antifungal resistance is high, several minimal molecular laboratory tools available.

3.2. *Candida krusei*

Candida krusei was first discovered in 1839 by Langenbeck from a patient with typhus, 75 years later Castellani proposed the suggestion that *C. krusei* may cause disease in humans [39]. Since then, it has been generally considered as a commensal in warm-blooded animals with low pathogenicity and virulence. In humans, *C. krusei* is generally considered to be a transient commensal and is infrequently isolated from mucosal surfaces. However, since 1960s there has been an increase in the number of reports of *C. krusei* as a human pathogen.

In contrast to most other ovoid shaped Candida spp., *C. krusei* cells are generally elongated in a feature similar to *C. kefyr* (formerly known as *C. pseudotropicalis*) among clinically important Candida spp. *C. krusei* has various colony morphologies. It has a multilayered cell wall consisting of an outer irregular coat of flocculent material, an electron-dense zone, a granular layer, a less granular layer, a thin layer of dense granules and another sparsely granular layer outside the cell membrane. The mannan component of the *C. krusei* cell wall has been shown to be different from other Candida spp. in containing (1–2) and (1–6) side chains in the ratio of 3:1 as being lightly branched [40]. Such differences may account for the variable behavior of *C. krusei* in biological fluids such as saliva and bronchial lavage fluid comparing with other Candida spp.

C. krusei has two basic morphological forms, yeast and pseudohyphae and both are often present simultaneously in growing cultures and not easily separated. *C. krusei* grows at a 37°C but can withstand temperature up to 45°C. *C. krusei* can grow in vitamin-free media even though most common Candida spp. require biotin or additional vitamin for growth. *C. krusei* ferments and assimilates glucose only as carbohydrate [39].

Like *C. auris*, *C. krusei* can adhere to abiotic surfaces but not to the same extent as *C. albicans*. Although adhesion to host surfaces is essential for colonization and invasion, *C. krusei* is able to colonize readily to inert surfaces such as implants and catheters by virtue of its cell surface hydrophobicity. Less pathogenic species—*C. parapsilosis*, *C. pseudotropicalis* (now *C. kefyr*) and *C. glabrata*—usually produce significantly less biofilm than the more pathogenic *C. albicans*,

but *C. krusei* produced the most extensive biofilm on the surfaces of polyvinyl chloride catheter disks regardless of the growth medium. This could demonstrate the very high cell surface hydrophobicity, and adherence of *C. krusei* to inert plastic surfaces, which may then have other species, facilitated biofilm development [39]. *C. krusei* does not adhere to buccal epithelial cells whereas *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, and *C. kefyr* do.

The susceptibility to lysozyme, an antimicrobial enzyme produced in phagosomes has been used as a method to assess the microbial virulence. Such tests indicate that the susceptibility to lysozyme of *C. krusei* > *C. parapsilosis* > *C. tropicalis* > *C. guilliermondii* > *C. albicans* > *C. glabrata*, the latter being the most resistant to lysozyme [41]. Interesting when pre-incubated in sucrose-supplemented media, *C. krusei* becomes highly sensitive to lysozyme as compared to *C. albicans*.

Pathogen profile: Diploid, does not belong to CTG clade, genome sequence available, antifungal resistance is high, minimal molecular laboratory tools available.

3.3. *Candida kefyr*

C. kefyr was first found in dairy products such as fermented milk, cheese, and yoghurt [42]. It was first isolated from a *C. kefyr* sample in 1909, named *Saccharomyces fragilis* at first, then *C. pseudotropicalis* and was reclassified as *Kluyveromyces marxianus* [43].

C. kefyr is rarely associated with disease [44] representing ~1% isolates of *Candida* spp. from clinical specimens [7, 45] (**Figure 1**). The first case of invasive *C. kefyr* (*C. pseudotropicalis*) was identified in a 58-year-old female with metastatic adenocarcinoma of the breast [46]. It has been reported to colonize oral cavities, gastrointestinal tract, and urinary tract. All infected patients were immunocompromised and had several potential risk factors [43] . There are only a few published cases of invasive *C. kefyr* infections.

These emerging pathogens of the Candida species themselves are typically not more virulent than *C. albicans*. It is generally thought that their conversion from commensalism to parasitism is largely determined by the host immune status [39]. In some cases these pathogens are resistant to multiple antifungal agents. For example, >90% of the recently emergent *C. auris* isolates are resistant to fluconazole, >30% are resistant to amphotericin B, and >5% to echinocandins, and >40% are resistant to classes of antifungal agents, while 4% are resistant to all three classes of antifungals available [35].

Pathogen profile: Ploidy is not determined, does not belong to CTG clade, Genome sequence available, antifungal resistance is moderate, few molecular laboratory tools available.

4. Other Candida species

Candida lusitaniae was firstly isolated from warm-blooded animals and was shown to cause opportunistic infections in humans in 1979 [47]. It is distinguished from other Candida species by its resistance to Amphotericin B however resistance profile *in vivo* is similar to

other Candida species. Like most pathogens of the Candida species, *C. lusitaniae* has similar ability to colonize individuals but can opportunistically infected in immune-compromised patients [48].

Pathogen profile: Haloid, belongs to CTG clade, genome sequence available, antifungal resistance is moderate—high, few molecular laboratory tools available.

Candida tropicalis is another prevalent NAC pathogen in Candida species. In immunocompromised mice and human patients, *C. tropicalis* isolates appeared to have increased virulence. Secreted aspartyl proteinase 5 and 9 (Sap5 and Sap9) antigens are expressed by *C. tropicalis*. Invasive *C. tropicalis* infections were found more frequently in acute leukemia or bone marrow transplants patients may indicate that polymorphonuclear leukocytes are the first defense line against of *C. tropicalis* [49]. Overexpression of *ERG11* gene mutations in *C. tropicalis* likely causes resistance to azoles.

Pathogen profile: Diploid, belongs to CTG clade, genome sequence available, antifungal resistance is moderate, several molecular laboratory tools available.

Candida dubliniensis is a species of chlamydospore- and germ tube-positive yeast, primarily recovered from HIV-infected individuals and AIDS patients. It has been shown to grow well at temperatures ranging between 30 and 37°C but not 42°C. *C. dubliniensis* is unable to express beta-glucosidase activity [50].

Pathogen profile: Diploid, belongs to CTG clade, genome sequence available, antifungal resistance is moderate, several molecular laboratory tools available.

Candida parapsilosis has increased in significance and prevalence over the past 2 decades. The infections are mainly associated with prosthetic devices and catheters, especially in the nosocomial spread. Risk factors of *C. parapsilosis* infections include the hydrolytic enzymes secretion, prosthetics adhesion, and biofilm formation [51].

Pathogen profile: Diploid, belongs to CTG clade, genome sequence available, antifungal resistance is moderate-high, several molecular laboratory tools available.

Candida guilliermondii is the sixth frequently isolated Candida species, an emerging pathogen in Latin America that rarely causes invasive candida infections. However, it has been reported to exhibits reduced susceptibility to fluconazole [52] thus further study of the anti-drug mechanism is required.

Pathogen profile: Haploid, belongs to CTG clade, genome sequence available, antifungal resistance is high, several molecular laboratory tools available.

Candida lypolytica (also known as *Yarrowia Lipolytica*) isolates formed narrow, multi-branched, true hyphae on cornmeal-Tween 80 agar [53]. *C. lipolytica* is a weakly virulent pathogen that is most clearly vascular catheter-related. It is sensitive to Amphotericin B and Ketoconazole *in vitro*.

Pathogen profile: Haploid, does not belongs to CTG clade [54], genome sequence partially available, antifungal resistance is moderate [55], few molecular laboratory tools available.

Candida rugosa rarely causes invasive infections; however, recently, isolates have been shown to be an increasing cause of fungal infections especially in Latin America. Besides, *C. rugosa* appears decreased susceptibility to fluconazole with various patterns following geographic regions [56].

Pathogen profile: Haploid, belongs to CTG clade, genome sequence is not available, antifungal resistance is high, few molecular laboratory tools available.

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Conflict of interest

There is no conflict of interest concerning this chapter.

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Chapter 2

Antifungal Activity of Brazilian Medicinal Plants against *Candida* Species

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Additional information is available at the end of the chapter

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Abstract

Due to the resistance of *Candida* sp. to the usual antifungal, the demand for active principles found in the plants has been the target of diverse studies around the world. There are few *in vivo* and human studies on the antifungal activity of medicinal plants in the mouth. Native and imported medicinal plants, used by the Brazilian population for traditional medicine use, are the subject of study in this chapter. Thirty-eight Brazilian plants were related to information on species, family, name, used part, and medical indication of popular use. All the species mentioned had their extracts tested *in vitro* against *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, among other species that occur more frequently in the mouth. In the articles consulted, there is a great variation in *Candida* species tested and in minimum inhibitory concentration. The *in vitro* studies serve as information for the continuity of studies on the best performing plants, validate the popular belief about the use, and provide subsidies for the development of new products that are effective in the control of oral and systemic candidiasis and that are cheap and accessible for the population.

Keywords: Brazilian medicinal plants, *Candida* sp., oral candidiasis, minimum inhibitory concentration

1. Oral candidiasis—by Elizete Maria Rita Pereira/Vagner Rodrigues Santos

The most common fungal pathogens detected from the oral cavity are *Candida* sp. and their transition of harmless commensals to pathogenic microorganisms is often related to decrease immunity. Often, candidiasis occurs in a localized and superficial manner in the oral cavity,

but it can be systemic or invasive and even lead to death in immunocompromised individuals [1, 2]. Like other pathogenic fungi, *Candida* spp. present dimorphism in the yeast and pseudohyphatic forms. The hyphal form is associated with epithelial cell invasion and thus causing tissue damage [3]. *Candida* spp. has other virulence factors such as adherence, production of tissue-damaging hydrolytic enzymes such as proteases, and the biofilm formation in host tissue and in medical devices [4]. Several predisposing factors, local and systemic, may result in the transition from yeast Candida to the hyphal form (pathogenic). Local factors include the use of prostheses, corticosteroid inhalers, and xerostomia, while systemic factors include immunosuppressed states as human immunodeficiency virus—HIV for example. Psoriasis, recently, was described as a predisposing factor for oral candidiasis [5].

The type of *Candida* spp. most commonly isolated from the oral cavity is *C. albicans*, which occurs in both healthy and diseased individuals [1, 6, 7]. Nonalbicans species were also isolated from the oral cavity of immunocompromised patients, such as *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, and *C. dubliniensis* [8]. Protective immune response can be induced by Candida in the host that allows its own survival. Immunocompetent adults, usually, present acquired immunity underlying the fungus that prevents the progression of oral colonization to symptomatic infection. Integrity of the mucosa is important for oral health because normally it prevents the invasion of microorganisms, as well as macromolecules, which may be antigenic [9].

1.1. Main types, diagnosis, and treatment of oral candidiasis

Candidiasis is an acute or chronic infection produced by *Candida* sp., often, limited to the skin and mucous membranes, but can produce a severe systemic disease in immunocompromised patients [3]. It may exhibit various clinical patterns such as the four primary oral forms: pseudomembranous candidiasis (thrush), acute erythematous oral candidiasis, chronic erythematous oral candidiasis, and chronic hyperplastic candidiasis (**Table 1**). These primary forms of candidiasis are also associated with lesions called secondary forms of candidiasis, such as angular cheilitis, median rhomboid glossitis, and *Candida* sp.-associated prosthesis stomatitis [10].

Diagnosis of oral candidiasis is established by identification of clinical signs and symptoms in conjunction with the presence of Candida organisms in the examination of an injury smear, biopsy examination containing hyphal form in the epithelium (Schiff's Periodic Acid), or positive culture and serological tests [11–13].

Generally, the drugs of choice for localized, uncomplicated candidiasis in patients with normal immune function are topical antifungal agents (**Figure 1**). These agents can achieve elevated levels of concentration in the oral epithelium [14]. Azoles act inhibiting the lanosterol 14-α-demethylase (enzyme involved in ergosterol biosynthesis) activity 14-α-demethylase (enzyme involved in ergosterol biosynthesis) and disrupt the cell membrane. The resistance to azole, generally, can be observed in HIV/AIDS patients receiving treatment for pre-HAART oral or esophageal candidiasis, for example. The resistance mechanisms of *C. albicans* include mutations that result in increased expression of efflux pumps (CDR1P, CDR2P, and MDR1P) and mutations in the target ERG11 drug [15].

Polyenes act by direct binding to ergosterol within the membranes of fungal cells, therefore, inducing the leakage of cytoplasmic content and leading to the death of microorganism.

Primary forms of oral candidiasis	Characteristics
Pseudomembranous candidiasis (oral thrush)	Confluent white plaques with epithelial desquamation and accumulation of keratin, fibrin, necrotic tissue, and fungal hyphae [5, 11]
	• Ability to easily remove white plaques with gauze [5]
	• Most commonly found in immunosuppressed individuals [5]
Acute erythematous oral candidiasis	• Known as "sore mouth antibiotic," due to its association with prolonged use of broad-spectrum antibiotics [11]
	• It presents as erythematous patches, commonly on the palate, but may also present on the buccal mucosa or on the back of the tongue [5]
Chronic erythematous oral candidiasis	• These are atopic lesions associated with angular cheilitis and prosthetic stomatitis [5]
	• Prevalence in HIV-positive and AIDS patients [11]
Chronic hyperplastic candidiasis (candida leukoplakia)	• Homogeneous type of adherent white or erythematous nodular/speckled type that cannot be easily erased [5, 11]
	• It is present bilaterally in the commissure regions of the buccal mucosa [11]

Table 1. Primary forms of oral candidiasis [10].

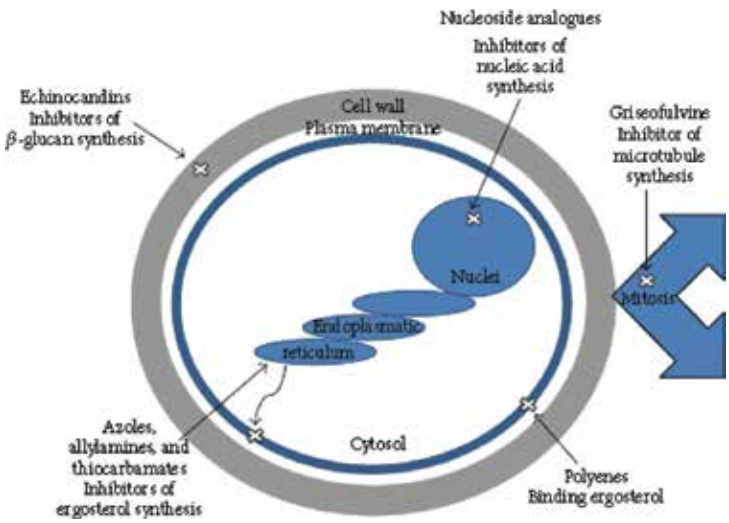

Figure 1. Primary targets of several antifungal agents by Garcia-Cuesta et al. [14].

Formulations of nystatin or amphotericin B are used for 4 weeks [11, 14]. Resistance was observed in case reports of cancer patients on chemotherapy and those who have received long-term prophylactic therapy. The mechanism of Candida resistence to the polyenes are not yet known, but seem to involve changes in the cell membrane composition [15].

Echinocandins are noncompetitive inhibitors of the β-1,3 glucan synthase encoded by the FKS1 gene of *C. albicans*, which leads to the formation of fungal cell walls with impaired structural integrity, leading to osmotic lysis. Resistance is associated with acquired or intrinsic

FKS1 point mutations [15]. Flucidosycin is a pyrimidine analogue, which is transported to fungal cells by cytosine permeases. After this, 5-fluorouracil and phosphorylation of 5-fluorodeoxyuridine monophosphate are deaminated. This nucleotide acts by inhibiting thymidylate synthase and interferes with DNA synthesis [14]. Mutations in the cytosine-permease FCY2 gene or the cytosine-deaminase gene FCY1 are the most common causes of drug resistance. To avoid this increased resistance, flucytosine is almost always administered to patients in conjunction with amphotericin B [15]. Griseofulvin, first isolated from griseofulvin, first isolated from *Penicillium griseofulvum,* inhibits the fungal mitosis to act by disrupting the production of microtubules in the spindle and cytoplasm [14].

Antifungal systemic agents are indicated in fungemia when found in low immunity or immunodeficiency, high agranulocytosis, cancer patients or patients with intravenous catheters [11]. Worldwide, an increase in the number of antifungal resistant yeasts is recognized. An important factor in contribution to human candidiasis is the ability of *Candida* species to form drug-resistant biofilms [4]. The antifungal resistance can occur by different mechanisms, such as the reduction of the intracellular accumulation of the drug, decreased affinity of the target by the drug, and neutralizing the effect of the drug. Depending on the mode of action of the antifungal compounds, the mechanism of resistance will be different [14].

The search for new antifungal agents and the characterization of new targets that are more appropriate and efficient have been proposed [4]. Potential alternative therapies include the use of new active principles obtained from different general sources, such as natural products, in particular, the plants that contain several components that are important sources of biologically active molecules [14].

2. Brazilian medicinal plants tested against *Candida* spp.—by Vagner Rodrigues Santos

2.1. Introduction

The search for therapeutic applications of medicinal plants and their derivatives has grown in the past years throughout the world. Several studies have been carried out in order to evaluate new biological properties from the biodiversity. The discovery of new antimicrobial components is of great relevance, particularly for dentistry, since bacterial and fungal infections of the oral cavity are a relatively common problem: *Candida albicans* is an opportunistic yeast commonly identified in denture stomatitis and other oral candidosis clinical forms [12].

These are examples of infectious conditions of the mouth, and the resistance to antimicrobials in clinical cases has stimulated the search for natural agents as alternative treatments for the mouth infectious conditions. In Brazil, local communities use plants and their extracts for different medicinal purposes and take advantage of the availability of these plants and the low cost for product preparation. Plants have been used as antimicrobial, anti-inflammatory, wounds scarring, and antihemorrhagic agents, just to mention a few [16].

Medicinal plants continue to be widely used in rural and urban areas of Brazil. However, the intense miscegenation of crops over the last few centuries has more popularized the use of

exotic native Brazilian plants and plants imported from other countries in popular medicine, especially in the southcentral part of the country. Most of these species were introduced by the Europeans and Africans, and are usually used according to the traditions of their places of origin [15, 16]. The growth of the pharmaceutical industry during the second half of the last century also distanced the Brazilian population from traditional medicine based on native plants. In the mid-1970s, for example, commercial pharmacies had lost their importance as the pharmaceutical industry completely dominated the drug market. This period was also marked by intense repression of mysticism, including the traditional use of medicinal plants. These facts are aggravated also by the continuous destruction of the rich Brazilian ecosystems, a process initiated with the exploration of Brazilwood by the Portuguese. As a consequence, remedies prepared with native plants, especially those of Amerindian origin, are now little known or used [16].

The Brazilian territory has about 20% of the world's biodiversity, including plants, which serve as raw materials for the production of herbal medicines and other products. The great cultural and ethnic diversity of Brazil is responsible for the knowledge transmitted over generations on the management and use of medicinal plants [17]. The high frequency of infections by the *Candida* species, as well as the occurrence of resistance to the usual antifungal, either in the hospital environment or in domestic use, as well as the increasing number of immunocompromised patients puts us in check and leads us to search for new active principles originated from medicinal plants that are effective in the control of microorganisms [18].

Several plants have been studied in Brazil based on popular use, mainly by rural communities [16].

The antifungal activity observed in some plants may be related to the presence of flavonoid glycosides and tannins, components that have antimicrobial and anti-inflammatory properties [19]. There is a growing interest in the use of tannins as antimicrobial agents. The activity of tannins against bacteria and yeasts can be measured by their action on the membranes, since they can cross the cell wall, composed of polysaccharides and proteins, and bind to its surface [20].

Studies with natural products generate difficulties regarding the comparison of results. This situation is due to the different presentations of the products used as tincture, ethanolic extract, aqueous extract, essential oil, among others, as well as the various methodological criteria employed [21] and also the different forms of phytotherapic presentation, among them, oral solutions, gel, and tea by decoction. The greater or lesser biological activity of the essential oils has been shown to be dependent on the composition of their chemical constituents, such as citral, pinene, cineole, caryophyllene, elemeno, furanodiene, limonene, eugenol, eucalyptol, and carvacrol. These constituents are responsible for the antiseptic, antibacterial, antifungal, and antiparasitic properties [22].

The mode of extraction of the active principles can influence significantly the antimicrobial activity. Biosynthesis of the constituents of a plant is strongly affected by the environment, harvest and postharvest, rainfall, temperature, luminosity, and humidity [23].

The mechanisms of action of medical plant extents on *Candida* spp. are still poorly studied. Several mechanisms of action have been proposed from the rupture of the cellular membranes, which several mechanisms of action have been proposed from the rupture of the cellular membranes, which seems to interrupt the cell cycle through the synthesis of proteins and alteration of the yeast DNA [24].

The most common microbiological methods for testing plant-derived products such as extracts, resins, and essential oils are agar diffusion tests and liquid-liquid tests such as macrodilution and microdilution [25]. The techniques of application of the plant antimicrobial substance in the diffusion method are by means of disc, stainless steel, or glass cylinders and agar perforation. The agar diffusion test, also called plaque diffusion, is a physical method in which a microorganism is challenged against a biologically active substance in solid culture medium and relates the size of the growth inhibition zone of the challenged microorganism [25, 26]. The application of the diffusion method is limited to fast-growing microorganisms, which are aerobic or anaerobic. The evaluation is comparative against a reference biological standard (positive control), and the zone or halo of inhibition of growth is measured starting from the circumference of the disc or well, to the margin where there is growth of microorganisms [27]. According to the size of the halo, the microorganisms can be classified as: sensitive, when the diameter of the zone of inhibition is greater or no more than 3 mm less than the positive control; moderately sensitive, halo greater than 2 mm, but smaller than the positive control of more than 3 mm; and resistant, diameter equal to or less than 2 mm. As a positive control, a standard antimicrobial is used, and as a negative control, the solvent is used for the dissolution of the extracts [28–32]. The recommended incubation conditions are 35–37°C for bacteria for 24 to 48 hours and for fungi from 25 to 27°C for 48 to 72 hours [33–39]. These tests serve to define the minimum inhibitory concentration that quantifies the lowest concentration of the product capable of inhibiting the growth of microorganisms [40–46] (**Table 3**).

Table 2 shows the species, families, popular names, and used parts of plants for the various applications in traditional medicine.

Herbs	Family	Local popular name	Used source	Medical use	Ref.
Allium sativum L.	Liliaceae	Garlic	Bulb	Antimicrobial, healing, antioxidant, antitumor	[47, 48]
Anacardium humile L.	Anacardiaceae	Cajuzinho-do-cerrado, little cuckoo	Shells, sheets, pulp	Antifungal, anti-inflammatory, hypoglycemic antioxidant, antimicrobial antiparasitic	[49, 50]
Anadenanthera colubrina (Vell) Brenan	Fabaceae	Angico branco, white Angico	Shells, resin	Healing, anti-inflammatory, antimicrobial	[51, 52]
Annona crassiflora Mart.	Annonaceae	Araticum	Shells, sheets, fruits	Antimicrobial cytotoxicity	[53, 54]

Herbs	Family	Local popular name	Used source	Medical use	Ref.
Arrabidaea chica (Hum. & Bonpl.) B. Verlot	Bignoniaceae	Crajiru	Sheets, shells	Anti-inflammatory, Antimicrobial Antihypertensive, antitumoral	[55, 56]
Azadirachta indica A. Juss	Meliaceae	Neem, nim	Oil, flowers, leaves, seeds, bark	Antimicrobial, insecticide, antimalarial	[57, 59]
Baccharis dracunculifolia DC	Asteraceae	Rosemary, broom	Sheets, flowers, stalk	Antimicrobial antioxidant, antitumoral, healing	[38, 60, 61]
Baccharis trimera (Less.) DC	Asteraceae	Carqueja	Flowers sheets oil	Antioxidant, antihepatotoxic, anti-inflammatory	[62, 63]
Calendula officinalis L.	Asteraceae	Calendula	Flowers	Anti-inflammatory, healing, antimicrobial	[64, 65]
Ceiba speciosa (A.St-Hil) Ravena	Malvaceae	Paineira	Shells, sheets	Antiematism, antihypertensive, antimicrobial	[66, 67]
Centaurium erythraea Rafn	Gentianaceae	Centaurea	Shells, sheets	Digestive, emetic, febrifuge, hepatic, antioxidant, anti-inflammatory	[49, 68]
Chrysobalanus icaco L.	Chrysobalanus	Ajiru	Sheets	Antimicrobial, anti-inflammatory, antitumoral	[71]
Coriandrum sativum L.	Apiaceae	Coriander, coentro	Sheets, seeds	Antibacterial, antioxidant, hepatoprotective, anticonvulsivant	[69, 70]
Croton campestris (A. St-Hill.)	Euphorbiaceae	Canopy, velame	Oil, barks, root	Anti-inflammatory, antimicrobial, antioxidant	[49, 72]
Curatella americana L.	Dilleniaceae	Sambaiba	Sheets	Antimicrobial, anti-inflammatory, antiulcerogenic, antihypertensive	[73, 74]

Herbs	Family	Local popular name	Used source	Medical use	Ref.
Dalbergia ecastophyllum (Linn.) Taub.	Leguminosae	Rabo-de-bugio	Resin, sheets	Antitumoral, antimicrobial, antioxidant, anti-inflammatory	[75, 76]
Drimys winteri (J.R.Forst & G. Forst)	Winteraceae	Casca d'anta	Bark	Antifungal, antibacterial, antioxidant	[77, 78]
Eugenia dysenterica ex DC Mart.	Myrtaceae	Cagaita	Leaves, barks	Antidiarrhoeic, antileukemic	[79, 80]
Eugenia uniflora L.	Myrtaceae	Pitanga	Leaves	Diarrhea, fever, diabetes, inflammation, headache	[81, 82]
Equisetum arvense L.	Equisetaceae	horsetail	Sheets, bark	Antioxidant, anti-inflammatory, antimicrobial, antitumoral	[83, 84]
Glycyrrhiza glabra L.	Fabaceae	Licorice Alcacuz	Root, rhizome	Antioxidant, anti-inflammatory, antiosteoporotic	[85, 86]
Hymenaea courbaril L.	Leguminosae	Jatobá	Sap, peel	Antimicrobial, anti-inflammatory, bronchitis, antidiarrheal	[87, 88]
Jacaranda cuspidifolia Mart.	Bignoniaceae	Rosewood Jacarandá	Barks, leaves, resin	Antimicrobial anti-inflammatory, antitumor	[89, 90]
Lafoensia pacari (A.St-Hill).	Lythraceae	Mangava brava	Stem bark	Anti-inflammatory analgesic	[91, 49]
Lippia sidoides Cham.	Verbenaceae	Rosemary-pepper, Alecrim-pimenta	Leaves, barks	Antinociceptive, anti-inflammatory antimicrobial	[60, 92]
Malva sylvestris L.	Malvaceae	Mauve, malva	Sheets, flowers	Cough, anti-inflammatory healing	[93, 94]
Maytenus salicifolia Mart Ex Reissek	Celastraceae	Holy Thorn, Espinheira santa	Sheets	Antiseptic, dyspepsia, antiulcer	[95, 96]
Melaleuca alternifolia Cheel	Myrtaceae	Melaleuca	Essential oil	Antiseptic anti-inflammatory, antifungal	[87, 97, 98]

Herbs	Family	Local popular name	Used source	Medical use	Ref.
Mentha piperita L.	Lamiaceae	Peppermint, H ortelã--pimenta	Sheets	Expectorant, carminative, anti-inflammatory antimicrobial	[55, 92]
Myroxylon peruiferum L.f.	Fabaceae	Cabreúva	Bark, fruits	Anti-inflammatory, anti-headache, antifungal	[87, 99]
Psidium guajava L.	Myrtaceae	Guava tree goiabeira	Leaves, fruits	Antioxidant, antimicrobial, anti-inflammatory	[81, 100]
Punica granatum L.	Punicaceae	Pomegranate Romã	Bark, peel, pericarp, leaves, juice	Antioxidant, anti-inflammatory, antimicrobial, anticarcinogenic	[20, 101, 102]
Ricinus communis L.	Euphorbiaceae	Castor mamona	Aerial parts	Antidiabetic, antifertility, anti-inflammatory, antimicrobial, antioxidant	[103, 104]
Sapindus saponaria var. drummondii (Hook. & Arn.) L. Benson	Sapindaceae	Soapberry	Leaves, fruits, barks	Diuretics, expectorants antifungal, antioxidant	[105, 106]
Schinus terebinthifolius Raddi	Anacardiaceae	Aroeira	Fruits, leaves, stem bark, essential oil	Antioxidant, anti-inflammation antimicrobial, antifungal, antiulcer	[69, 107]
Stryphnodendron adstringens (Mart) Coville, 1910	Fabaceae	Barbatimão	Bark leaves	Anti-inflammatory, cicatrizant, antimicrobial	[108–110]
Vismia guianensis (Aubl.) Pers.	Clusiaceae	Sealing wax Pau-de-lacre,	Resin, sheets, stalk	Anti-inflammatory, antifungal	[87, 111, 112]
Ziziphus joazeiro Mart.	Rhamnaceae	Juazeiro	Leaves, fruits, bark, root.	Anti-inflammation, antimicrobial, healing	[87, 113]

The table lists the native species and those imported or brought to Brazil.
*Ref.—References.

Table 2. Relation of Brazilian medicinal plants tested *in vitro* against *Candida* species.

Herbs	Active compounds	Microorganism	MIC: µg//mL	Ref.
Allium sativum L.	Quercetin, cyanidin,	*C. albicans*	0.125	[47, 48]
	allistatin, allicin, ajoene	*C. glabrata*	0.312	
		C. tropicalis	1.56	
		C. parapsilosis	12.5	
Anacardium humile	Tannins,	*C. albicans*	1.50	[114, 49, 50]
	saponins, flavonoids amentoflavone			
Anadenanthera colubrina (Vell)	Tannins, flavonoids	*C. albicans*	0.031	[115, 51, 52]
Annona crassiflora(Mart.)	Antioxidant, tannins	*C. albicans*	2.0	[54, 116]
		C. tropicalis	0.25	
		C. krusei	0.5	
Arrabidaea chica	Isoscutellarein, 6-hydroxyluteolin, hispidulin, scutellarein, luteolin, apigenin; anthocyanins, coumarins, flavonoids, saponins, tannins, triterpenes	*C. albicans,*	0.007/0.015	[55, 56, 102]
		C. dubliniensis		
		C. parapsilosis		
		C. tropicalis		
		C. krusei		
		C. guilliermondii,		
		C. utilis		
		C. lusitaniae		
		C. glabrata		
		C. rugosa		
Azadirachta indica	Nimonol, mahmoodin, naheedin	*C. albicans*	1000/500	[58, 117, 118]
Baccharis dracunculifolia	Artepillin C, baccharin, kaempferide, drupanin, p-coumaric acid, culifolin, caffeic acid phenethyl ester, chlorogenic acid, kaempferol, pinocembrin, naringenin, chrysin	*C. albicans,*	0.350	[24, 38, 92, 119]
		C. glabrata.	0.43	
		C. albicans,	20–320	
		C. tropicalis,		
		C. stellatoidea,		
		C. krusei		
Baccharis trimera	Flavonoids, phenolic acids, quercetin, luteolin, nepetin or eupafolin, apigenin, hispidulin, phytoalexin	*C. albicans*	2.0	[120, 121]
Calendula officinalis L.	Quercetin, hyperosides, α-cadinol, gamma-cadinene, 1,2,3-cadinatriene, α-muurolol	*C. albicans*	11.0 a 30	[64, 65]
		C. parapsilosis		
		C. dubliniensis		
		C. glabrata		
Ceiba speciosa (A.St-Hil) Ravena	Quercetin, ruthin, kaferol, gallic acid, chlorogenic acid, elagenic acid, caffeic acid	*C. albicans*	0.2	[66, 67]

Herbs	Active compounds	Microorganism	MIC: µg//mL	Ref.
Centaurium erythraea Rafn	Erytaurin, gentiopicrin, erytro-centaurin	*C. albicans,*	10.5	[49, 68]
Coriandrum sativum L.	Decanal, trans-2-decenal, 2-decen-1-ol, cyclodecane, mono- and sesquiterpene hydrocarbons	*C. albicans*	0.007	[60, 92, 122]
		C. tropicalis	20	
		C. stellatoidea	32	
		C. krusei	20	
Chrysobalanus icaco	Pomolic acid	*C. albicans,*	1.56	[71]
		C. tropicalis	6.25	
Croton campestris L.	Spathulenol, borneol,	*C. albicans*	5.25	[49, 123, 124]
	B-caryophyllene,			
	1,8-cineole			
Curatella americana L.	Tannins, 4-O-methyl—catechin; epicatechin-3-O-gallate; 4-O-methyl-catechin-3-O-gallate	*C. albicans*	15.6	[73]
		C. tropicalis	31.3	
		C. parapsilosis	31.3	
Dalbergia ecastaphyllum	Luteolin, quercetin,	*C. albicans,*	64	[125, 126]
	biochanin A,	*C. glabrata,*		
	Dalbergin, liquiritigenin,	*C. tropicalis*		
	rutin			
Drimys winteri	Polygodial,	*C. albicans*	0.015	[92, 127]
	caffeic acid,			
	3-caffeoylquinic acid			
Eugenia dysenterica ex DC Mart.	Caryophyllene,	*C. albicans,*	20	[69, 92]
	bicyclogermacrene,	*C. tropicalis,*	32	
	spathulenol,	*C. stellatoidea,*	32	
	Caryophyllene oxide	*C. krusei.*	20	
		C. albicans	0.250	
Eugenia uniflora Linn	Castor oil,	*C. krusei*	250	[79, 80, 128]
	isoquercetin,	*C. famata,*	125	
	quercetin	*C. guilliermondii*	500	
		C. tropicalis	125	
Equisetum arvense L.	Camptothin A,	*C. albicans*	0.250	[129, 81, 82, 130]
	Eugiflorins D1 and D2,	*C. albicans*	1000	
	afzelin, myricitrin, quercetin, myricetin, betulinic acid, centelloside C	*C. tropicalis*	31.2	
		C. krusei	31.2	
		C. parapsilosis	125	
Glycyrrhiza glabra L.	Flavonoids, phenolic acids, alkaloids, phytosterols, tannins, and triterpenoids	*C. albicans*	0.78–3.12	[131, 132]

Herbs	Active compounds	Microorganism	MIC: µg//mL	Ref.
Hymenaea courbaril L.	Triterpenic saponins, glycyrrhizin, glabridin	*C. glabrata*	8	[133]
Jacaranda cuspidifolia Mart.	Terpene, phenolic, salicylic acid	*C. albicans*	1.25	[87]
		C. glabrata	0.625	
		C. krusei	1.25	
		C. parapsilosis	1.25	
		C. tropicalis	0.625	
Lafoensia pacari St. Hil.	Saponins, coumarins, quinones, flavonoids, tannins, triterpenes, steroids, alkaloids	*C. albicans*	16	[90]
Lippia sidoides Cham.	Ellagic acid	*C. albicans,*	4.40	[60, 92]
Malva sylvestris L.	Isoborneol, bornyl acetate, α-humulene, α-fenchene	*C. albicans*	0.250	[92, 134]
Maytenus salicifolia Mart Ex Reiss	Mucopolysaccharides, mucilages, flavonoids	*C. albicans*	20	[69, 93, 94]
		C. tropicalis	32	
		C. stellatoidea	32	
		C. krusei	20	
Melaleuca alternifolia Cheel	Tannins, nepeticin, rigidenol, gliquidone, 11-Î ± –hydroxygliquidone, 16-b-hydroxypristymerin	*C. albicans*	50	[95, 96]
Mentha piperita L.	8-cineole, neomenthol, menthol, carvone, acetato de metila, trans-cariofileno e viridiflorol menthol, menthone	*C. albicans*	0.73	[87, 97, 98]
Myroxylon peruiferum	α-Copaene, safrole, δ-cadinene, cumarin, cabreuvina	*C. albicans*	0.500	[55, 92]
Psidium guajava	Phenolic, flavonoid, carotenoid, terpenoid triterpene	*C. albicans*	1.25	[87, 99, 135]
		C. glabrata	1.25 µg	
		C. krusei	0.625	
		C. parapsilosis	0.625	
		C. tropicalis	1.25	
Punica granatum	Tannins, piperidine alkaloids, polyphenols, oxalic acid, malic acid, ascorbic acid, estrone punicic acid, punicalagin	*C. albicans*	20	[136, 69, 81]
		C. tropicalis	32	
		C. stellatoidea	20	
		C. krusei	32	
		C. albicans	125	
		C. krusei	15,6	

Herbs	Active compounds	Microorganism	MIC: µg//mL	Ref.
Ricinus communis	Ricinoleic acid,	*C. albicans,*	125	[137–139]
	ricin, ricinin	*C. dubliniensis,*	15.6	
		C. parapsilosis	125	
		C. tropicalis	12.5	
		C. krusei	125	
		C. guilliermondii	100	
		C. utilis,	100	
		C. lusitaniae,	30	
		C. glabrata,	100	
		C. rugosa	30	
Sapindus saponaria var. Drummondii (Hook. & Arn.) L. Benson	Terpene-acetylated saponins	*C. albicans*	200/400	[140, 141]
	hederagenin			
Schinus terebinthifolius Raddi	α-Pinene, sabinene,	*C. albicans*	300/600	[105, 106, 142]
	z-salven, β- pinene,	*C. parapsilosis*	600	
	α-funebrene,	*C. glabrata*	300	
	limonene, myrcene,	*C. tropicalis*	300	
	alphaphellandrene			
Stryphnodendron adstringens (Mart) Coville, 1910	Gallic acid,	*C. albicans,*	4.25; 20	[49, 143, 144]
	procyanidin	*C. tropicalis,*	32	
	tannins,	*C. stellatoidea,*	32	
	delphinidin	*C. krusei*	20	
		C. albicans	7.8	
Vismia guianensis (Aubl.) Pers.	Vismiofuranoxantona, isojacareubina	*C. albicans*	4.25	[108, 110, 145]
	flavan-3-ol: epicatequina			
Zizyphus joazeiro Mart.	Betulinic acid, oleanolic acid, caffeine, amphibine D, jujubogenin	*C. albicans*	0.625	[87, 92, 112, 146]
		C. glabrata	1.25	
		C. krusei	0.625	
		C. parapsilosis	1.25	
		C. tropicalis	1.25	
		C. albicans	1.00	

* Ref—References.

Table 3. Minimum inhibitory concentration(MIC)—*in vitro* antimicrobial test of Brazilian medicinal plants against *Candida* species.

3. Oral candidiasis clinical trial studies

There are few clinical studies in humans on the efficacy of extracts from Brazilian plants in the treatment of oral candidiasis. More recently, human effectiveness of Brazilian green propolis derived from *B. dracunculifolia* on plaque control and gingivitis [49] has been shown

for the prevention and control of oral mucositis and candidiasis [147, 148] and compared green propolis gel with benzydamine hydrochloride in cancer patients and irradiated in the head and neck regions [149]. Also, the Brazilian red propolis, derived from *D. ecastaphyllum*, both extract and gel, inhibited *C. albicans* in vitro, periodontopathogenic bacteria in vitro and in vivo, besides controlling periodontitis in rats [150]. The antifungal activity of Brazilian green propolis, originated by *Baccharis dracunculifolia*, was proven when tested against *Candida albicans* collected from HIV-patients mouth. The authors also demonstrated the more effectivity of propolis compared with usual antifungal tested [29].

4. Conclusions

In this chapter, there is information about the most used Brazilian species of plants against *Candida* species. The highest antimicrobial activities were obtained with diverse plant extracts. Some tests were done with the wild-type microorganisms collected from patients' mouths; however, most tests were done using standardized American Type Culture Collection (ATCC) samples. There is a certain difficulty in doing clinical tests on humans, especially when it comes to natural products. On the other hand, tested are antibacterial and anticandidal agents and could be used in the treatment of various oral diseases caused by multiresistant microbial agents. It is also clear from this study that the antifungal activity of these 38 medicinal plants was found with ethanolic, methanol, n-butanol, and chloroformic fractions. Studies have also revealed that the plants tested are not toxic at therapeutic doses with good antimicrobial properties. However, this study is an important step toward clinical evaluation in order to produce improved phytomedicine in the treatment of oral candidiasis for multiresistant *Candida albicans*.

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A. Plant image glossary index

Photo Credits (alphabetical order of Latin names): 1 Allium sativum, alho; 2 *Anacardium humile*, cajuzinho do campo. https://www.dicaparasaude.com/beneficios-do-cajui/; 3 *Anadenanthera colubrina* (Vell.) Brenan; Angico branco. https://sites.unicentro.br/wp/manejoflorestal/8598-2/; 4 *Annona sylvatica* (Araticum) http://independente.com.br/os-araticuns/; 5 *Arrabidea chica* (crajirú), https://manausalerta.com.br/pesquisa-analisa-acao-anti-inflamatoria-do-crajiru/, 6 *Azadirachta indica* (nem) -https://br.pinterest.com/pin/520095456944307200/, 7 *Baccharis dracunculifolia*, alecrim. http://www.ufrgs.br/fitoecologia/florars/open_sp.php?img=14749, 8 *Baccharis trimera* (carqueja) - http://plantaslujan-a.blogspot.com/2015/01/baccharis-trimera.html, 9 *Calendula officinalis*, calêndula. https://plantsam.com/calendula-officinalis/10 *Ceiba*

speciosa(https://www.ebay.co.uk/itm/50-seeds-of-Ceiba-speciosa-Chorisia-speciosa-bottle-of-fake-kapok-tree-R-/151774995083, 11 *Centaurium erythraea*, centaurea. http://naturalhealingroom.com/Centaury-Herb-Centaurium-Erythraea_p_1337.html, 12 https://en.wikipedia.org/wiki/Chrysobalanus_icaco; 13 *Coriandrum sativum*, coentro. https://commons.wikimedia.org/wiki/File:Starr_070906-8875_Coriandrum_sativum.jpg; 14 *Croton campestris*, https://www.flickr.com/photos/142712970@N03/38936756240; 15 *Curatella americana*, cajueiro-bravo-do-campo. https://pt.wikipedia.org/wiki/Cajueiro-bravo-do-campo; 16 Dalbergia ecastophyllum http://www.ufal.edu.br/unidadeacademica/ceca/pt-br/pos-graduacao/zootecnia/dissertacoes/talita-almeida-de-paula; 17 *Drimys winteri*, https://futureforests.ie/products/drimys-winteri; 18 *Equisetum arvense*, https://www.ebay.com/itm/Equisetum-Arvense-15-Fresh-Seeds-Herb-Medicinal-Plant-Field-Common-Horsetail-/132339802749; 19 *Eugenia dysenterica*, cagaita. http://www.viveiroipe.com.br/?mudas=cagaita; 20 *Eugenia uniflora*, pitangueira. https://www.fazfacil.com.br/jardim/pitangueira/; 21 Glycyrrhiza glabra https://www.indiamart.com/proddetail/glycyrrhiza-glabra-mulethi-extract-12502491912.html; 22 Hymenaea courbaril http://fredsonpaivareporter.blogspot.com/2017/01/hymenaea-courbaril-viveiro-da-patrulha.html; 23 Jacaranda cuspidifolia -https://br.pinterest.com/pin/24769866672935594/?lp=true; 26 *Malva sylvestris*, malva. http://johnstarnesurbanfarm.blogspot.com/2011/11/malva-sylvestris.

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Section 2

Adaptation and Resistance

Chapter 3

Carbon Sources Attribute to Pathogenicity in *Candida albicans*

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Additional information is available at the end of the chapter

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Abstract

This topic was to examine the impact of galactose or fructose upon the assimilation of secondary carbon sources by *Candida albicans*. *C. albicans ICL1* gene is repressed upon addition of 2% galactose or fructose to lactate- and oleic acid-grown cells. Further studies on Ca*FOX2*, Ca*FBP1* and Ca*MLS1* transcripts in response to galactose or fructose on assimilation of lactate and oleic acid resulted in repression of these genes. The Ca*ICL1* gene, which encode the glyoxylate cycles enzymes isocitrate lyase are required for growth on non-fermentable carbon sources. However, the enzyme CaIcl1 was not destabilized by galactose, but was degraded in response to fructose. In contrast, *S. cerevisiae* Icl1 has retained the molecular apparatus of protein degradation in response to either galactose or fructose. Screening of ubiquitination site by http://www.ubpred.org/ showed that *C. albicans* lacks ubiquitination site in gluconeogenic and glyoxylate cycles enzymes as compare to *S. cerevisiae*. Addition of a putative *S. cerevisiae* ubiquitination site carboxy terminus of CaIcl1 led to galactose- accelerated degradation of this protein in *C. albicans* cell via a ubiquitin-dependent process. In the other hand, CaIcl prior to addition of ubiquitination site was degraded upon exposure to fructose; addition of *S. cerevisiae* ubiquitination site to CaIcl1 further increased the speed of protein degradation.

Keywords: *Candida albicans*, galactose, fructose, glyoxylate cycles, isocitrate lyase, gluconeogenic, metabolic adaptation, protein degradation, ubiquitination

1. Introduction

Candida albicans is eukaryotic diploid (2N) sexual yeasts of the kingdom fungi [1]. *C. albicans* can divide asexually or can undergo parasexual reproduction, heterothallic or homothallic mating [2]. *C. albicans* genome sequencing project revealed the presence of sequences homologous to

the *Saccharomyces cerevisiae MAT* (mating type) loci, *C. albicans MTL* (mating-type-like) loci [3]. Members of the genus *Candida* are very heterogeneous, can grow in at least three different morphologies; yeast, pseudo-hyphae and hyphae, such as *C. albicans* and *C. dubliniensis* [4, 5]. Further morphological forms exist during colony switching, such as white-opaque switching system involves switch between white domed colonies and opaque flat colonies [6], mating type-like locus a (*MTLa*) and *MTLα* cells must switch from white to opaque to become mating-competent [7]. Opaque cells secreted pheromones resulting in the formation of conjugation tubes, and subsequently, cell and nuclear fusion occur to form tetraploid (4N) cells. Mating products can be induced to undergo concerted chromosome loss to return to the diploid state [8, 9].

In order to proliferate in a wide range of environmental niches, pathogens must not only depend on certain virulence factors, it is also important to have a flexible metabolism; therefore, they can assimilate a variety of carbon sources that are scarce or the only available carbon source at a specific environmental niche. The primary and preferred sources of metabolic carbon for most organisms are carbohydrates; it is used for generating energy and producing biomolecules. Before entering the glycolytic pathway, most sugars are converted to fructose-6-phosphate or glucose-6-phosphate. ATP and NADH are produced from the conversion of hexose phosphates into the key metabolite pyruvate in glycolysis pathway. From there, two major strategies of energy production (fermentation and respiration) are carried out by cells. Although NAD+ is regenerated by both processes, respiration is more efficient than fermentation as it produces additional ATP through the oxidative phosphorylation and tricarboxylic acid (TCA) cycle. Glycolysis is the central, common pathway for both processes and it is critical for carbon assimilation; the pathway has been shown to be up-regulated during infections and important to the virulence in pathogenic bacteria, parasites, and fungi [10–12]. Glycolysis, gluconeogenesis, and the glyoxylate cycle are part of *C. albicans* central metabolism.

Central metabolic pathway such as glycolysis is strictly regulated, transcript of glycolytic enzymes is regulated in response to environmental conditions such as carbon source and availability, oxygen levels, and to cellular demands such as energy needs and metabolite concentrations. However, the regulators of glycolytic gene expression in most species have not been identified; for eukaryotes understanding of transcriptional control of glycolysis is mainly based on the non-pathogenic yeast *Saccharomyces cerevisiae* [13]. The transcription regulators (Gcr1 and Gcr2) are responsible for inducing the expression of the glycolytic genes in *S. cerevisiae* [14, 15], Gcr1p binds to CT boxes (*5′-CTTCC-3′*) upstream of the glycolytic genes and Gcr2p acts as a co-activator by forming a complex with Gcr1p [16]. Inactivation of either gene (*GCR1* and *GCR2*) result in growth defects during culture on glucose, due to the decreasing expression levels of the glycolytic genes [15]. However, on non-fermentable carbon sources, the mutant strains display wild type growth rates [17]. Another glycolysis-specific regulator in *S. cerevisiae* is Tye7 (also known as Sgc1); it is involved in the activation of several glycolytic genes, although not to the extent of Gcr1 and Gcr2, this activation is independent of *GCR1.* No growth defects under any carbon source regime are detected in *tye7* mutant strain [18].

C. albicans is an opportunistic fungus that is capable of metabolizing carbon sources through respiration and fermentation pathway similar to that of a typical eukaryotic cell. The culprit for more than half of all *Candida* infections is *C. albicans* [19]; this shows the importance of studying the metabolism of this pathogen for the development of effective antifungal treatments. *C. albicans*

is a Crabtree-negative organism that lacks *GCR1/2* homologs and must control transcription of glycolytic genes differently as compared to *S. cerevisiae*. Tye7 and Gal4 are identified as two fungal-specific activators of the glycolytic pathway in *C. albicans*, severe growth defects were observed in the mutant strains (*tye7* and *gal4*) cultured on fermentable carbon sources when respiration was inhibited or oxygen was limited. Furthermore, chromatin immunoprecipitation coupled with microarray analysis (ChIP-CHIP) and transcription profiling showed these factors bind to and regulate expression of the glycolytic pathway genes. *C. albicans* mutant strains (*tye7* and *gal4*) showed attenuated virulence in *Galleria mellonella* infection model, therefore *TYE7* and *GAL4* genes are required for pathogenicity and virulence of *C. albicans* [20].

Gluconeogenesis is required for yeast cells to generate sugar phosphates for the synthesis of essential cellular components, during the growth on non-fermentable carbon sources. Under physiological conditions with two exceptions, most of the glycolytic reactions are reversible. In many yeasts including *S. cerevisiae* and *C. albicans*, due to unfavorable thermodynamic balances, pyruvate kinase and phosphofructokinase have to be bypassed, this is achieved by phosphoenolpyruvate carboxykinase (Pck1, building phosphoenolpyruvate), and fructose-1,6-bisphosphatase (Fbp1, building fructose-6-phosphate). The enzymes of the glyoxylate cycle are necessary for gluconeogenesis, such as malate synthase (*MLS*) and isocitrate lyase (*ICL*). Moreover, in *S. cerevisiae,* in other to replenish TCA cycle intermediate (anaplerotic reactions), the succinate/fumarate transporter, Acr1p, has been shown to be a link between the glyoxylate cycle and the TCA cycle [21]. Not much is known about gluconeogenesis in *C. albicans*, whereas carbon sources/sugar phosphate may become available during lymphatic and blood infection, on skin after incorporation in macrophage, gluconeogenesis and the glyoxylate cycle may be depended for providing carbohydrates for cell wall biosynthesis [22].

The glyoxylate cycle is a "modified tricarboxylic acid (TCA) cycle," instead of the two decarboxylation steps of the TCA cycle the key enzymes of the glyoxylate cycle (isocitrate lyase, *ICL1* and malate synthase, *MLS1*) convert isocitrate and Acetyl-CoA into succinate and malate. *ICL1* splits the isocitrate, C_6-unit into succinate and glyoxylate, which in turn is condensed by malate synthase, *MLS1* with Acetyl-CoA generating free CoA-SH and malate. Malate is then processed malate dehydrogenase to continue the cycle and succinate is released as net product, which can be used to replenish the TCA cycle or to function as precursors for carbohydrate biosynthesis or amino acid biosynthesis. C_4-units (succinate) are generated by converting two Acetyl-CoA units generated by various catabolic processes. Therefore, the glyoxylate cycle enable cells to utilize fatty acids or C_2-units such as ethanol or acetate as sole carbon source [23].

Fatty acids are broken down in the mitochondria by catabolic process (beta oxidation) to generate two-carbon units (Acetyl-CoA), which can be oxidized to CO_2 and H_2O via the TCA cycle or used to generate hexose via the glyoxylate and gluconeogenesis, and NADH and $FADH_2$, which are co-enzymes used in electron transport chain. In yeast *S. cerevisiae,* β-oxidation and peroxisome biogenesis are regulated by transcription factors, *OAF1* and *PIP2*. However, such homolog is not present in *C. albicans.* Instead, *C. albicans* share a single homolog of transcription factors FarA/FarB with *A. nidulans*, *CTF1* which regulate genes for β-oxidation enzymes, glyoxylate cycle, and the gluconeogenesis in the presence of fatty acids. *CTF1* is important for growth on fatty acids and regulation of several genes encoding enzymes associated with β-oxidation, including *FOX2*. Deletion of *FOX2* (*fox2*Δ/*fox2*Δ (*fox2*Δ/Δ) strains) confers a mild

attenuation of virulence [24, 25]. Therefore, *C. albicans* appears to share more similarity to that of filamentous fungi than to that of budding yeast at phenotypic and genotypic level in the regulation of alternative carbon assimilation pathways, but there are clearly *Candida*-specific adaptations in these regulatory networks [26].

Glyoxylate cycle is present in fungi but not in mammals, and β-oxidation of fatty acids develop in different directions between mammals and fungi. Therefore, in order to have a better understand on the importance of these pathways, physiological and virulence of mutant strains lacking of these genes encoding key enzymes the β-oxidation multifunctional protein (*FOX2*), glyoxylate cycle (isocitrate lyase, *ICL1*), and gluconeogenesis (fructose-1,6-bisphosphatase, *FBP1*) was studied. Deletion of *ICL1* or *FOX2* or *FBP1* confers attenuation to some degree in a mouse model of disseminated candidiasis. Therefore, confirming that alternative carbon sources are relevant nutrients in vivo, and *C. albicans* probably consume multiple carbon sources during infection. Finally, in vitro genomic and phenotypes analysis indicate that the regulatory networks that control alternative carbon metabolism in *S. cerevisiae* differ significantly from the paradigms developed in *C. albicans* [25, 27].

In the present of glucose, *C. albicans* undergoes yeast-to-hyphal transition which is crucial for virulence and invasion of host cells [28]. The hyphal form can diffuse through the tissues or form mycelial biofilms, and the yeast form is thought to disseminate easily via body fluids [29]. Glucose is an important carbon sources in central metabolism pathway, thus glucose sensing and response is closely regulated and highly evolved in most organisms. For example, *C. albicans* appears to have over 20 different hexose transporters that are expressed under different conditions and *S. cerevisiae* has at least 17 [30], it is presumed that this large number of hexose transporters is required because *S. cerevisiae* prefers to ferment glucose. *S. cerevisiae* demands a high influx of glucose because for each molecule of glucose metabolized, it yields only two molecules of ATP. In contrast, *C. albicans* also has many hexose transporters although it is presumed to prefer respiration and thus can yield up to 38 ATPs per glucose molecule metabolized [31]. This might be the reason why *C. albicans* can thrives in various niches, which likely release many different sugars as carbon sources.

Not much is known about how *C. albicans* senses and responds to sugars, therefore *S*. cerevisiae a paradigm for non-conventional yeasts is used as a model to elucidate sugar-sensing and signaling pathway that primarily regulates glucose transport. In yeast *S. cerevisiae*, glucose is detected by two glucose sensors in the cell membrane, which are Snf3 and Rgt2. The glucose sensors are orthologs of transporters with long cytoplasmic tails for intracellular signaling, which are missing from glucose transporters. The sensors cannot transport sugar but they control sugar acquisition by regulating the expression of genes encoding hexose transporters (*HXTs*) [32]. A single point mutation in the *S. cerevisiae* glucose sensors Snf3 (R229K) or Rgt2 (R231K) resulted in constitutive expression of genes encoding hexose transporters, because these mutations may convert the sensors into their glucose-bound (signaling) conformations [33]. *C. albicans* possesses glucose sensors, Hgt4 (orf19.5962) which is an ortholog of the Snf3 and Rgt2, Hgt4 generates an intracellular signal to induce expression of certain *HGT* genes encoding hexose transporters which is required for growth on glucose, for optimal virulence, and for filamentation [34].

Similar observation is observed between *S. cerevisiae* and *C. albicans*, *ICL1* and *PCK1*, whereby upon exposure to 2% glucose the transcription from DNA to mRNA are repressed. However, in contrast to *S. cerevisiae*, Icl1 and Pck1, which are degraded rapidly upon exposure to glucose. *C. albicans*, Icl1 and Pck1 remain stable [35]. This suggests that *C. albicans* undergoes catabolite inactivation on transcriptional level but not on the protein degradation level, which further implicate that the proteins (Icl1 and Pck1) are subjected to post-translational modifications. Post-translational modifications of proteins, such as acetylation, phosphorylation and ubiquitination, play important roles in generating and relaying signal in almost every cellular pathway as well as cellular adaptation of all organisms, which includes their growth, differentiation, division and development. These protein modifications are important for the regulation of the pre-existing proteins' functions, activities and stabilities, thereby controlling dynamic cellular processes [36].

Similar to that in *S. cerevisiae*, *C. albicans* polyubiquitin is encoded by the *UBI4* gene, which has three rather than five tandem repeats in a consecutive head-to-tail arrangement that is found in *S. cerevisiae* [37]. Decrease in the polyubiquitin encoded by the *UBI4* gene in *C. albicans* induces the growth of hyphae and pseudohyphae [38]. This was compatible with previous studies indicating that the ubiquitination via the E2 enzyme, Rad6 inhibits hyphal development in *C. albicans* [39]. Furthermore, polyubiquitin is presumed to contribute to stress responses in *C. albicans*, because downregulation of *UBI4* resulted in mild temperature sensitivity in stationary cells grown in glycerol, but not during growth on glucose [38]. Therefore, it has been implied that the *UBI4* gene is necessary for viability in *C. albicans* [38]. For example, recent studies show that ubiquitination sites are present in glyoxylate cycle and gluconeogenesis enzymes from *S. cerevisiae* but absent from their *C. albicans* homologs [35]. This indicates that evolutionary rewiring of ubiquitination targets upon glucose exposure; this implies *C. albicans* proteins remain functional, allowing it to continue metabolize alternative carbon sources [35]. This metabolic flexibility is presumed to be important during infection and colonization of dynamic host niches with variable carbon sources.

Lorenz and Fink [27] have proved the importance of key enzyme, *ICL1* of glyoxylate cycle to the virulence and pathogenicity of *C. albicans*. Deletion of the gene cause attenuated virulence in mice model. This highlights the importance of *ICL1* as a factor that contributes to the virulency and viability of *C. albicans* and provides a new target site for antifungal drugs test. For example, caffeic acid, rosmarinic acid, and apigenin were found to have antifungal activity against *C. albicans ICL1* when tested under glucose-depleted conditions [40].

Thus, it is important to have a better understanding and studying the mechanism involved, and the fitness attribute of the key enzymes in central metabolism of *C. albicans*. Recent studies by Sandai et al. [35] indicate that glucose trigger degradation of transcript in *C. albicans*, *ICL1* and *PCK1* but not the protein. This suggests that *C. albicans* have undergone evolutionary rewiring and lacks ubiquitination site as compared to *S. cerevisiae*. As such, this study was mainly focused on the effects of galactose and fructose on the central metabolism of *C. albicans*. Galactose is metabolized to the more metabolically useful glucose-6-phosphate by the enzymes of the Leloir pathway. This pathway is necessary as the initial enzymes of glycolysis are unable to recognize galactose. Of all the sugars found in nature, only D-fructose feeds directly into glycolysis, the central pathway of carbohydrate metabolism.

2. Effect of galactose or fructose on *C. albicans FOX2, FBP1, MLS1* and *ICL1* mRNAs

There are reports that state repression on transcriptome by glucose in *S. cerevisiae* [41]. For example, study by Yin et al. [42] using northern blotting and transcriptomic analyses showed that transcripts encoding the gluconeogenic enzymes (FBP1 and PCK1) are repressed by glucose in *S. cerevisiae*. Furthermore, previous work by Sandai et al. [35] reported that *ICL1* and *PCK1* are repressed by glucose in *C. albicans* and *S. cerevisiae*. To reconfirm this report and to compare it with the galactose and fructose responses of *C. albicans* more directly in this study, we first examined the responses of *S. cerevisiae* glyoxylate cycle (Sc*ICL* and Sc*MLS1*), β-oxidation (Sc*FOX2*) and gluconeogenesis (Sc*FBP1*) mRNAs using the following experimental approach.

S. cerevisiae ICL1 mRNA levels showed a dramatic decrease within 1 hour of galactose or fructose addition to the cells growing on lactate or oleic acid. Similarly, Sc*FBP1*, Sc*FOX2* and Sc*MLS1* mRNAs levels declined after galactose or fructose addition to the cells growing on lactate or oleic acid media. This strong repression occurred 1 hour after galactose or fructose addition. These confirmed that in *S. cerevisiae*, the *ICL1*, *FBP1*, *FOX2* and *MLS1* transcripts are strongly repressed by galactose and fructose [43].

Global transcriptional responses of *C. albicans* to low (0.01%), medium (0.1%) and high (1%) glucose concentrations by microarray analysis indicated that a total of 347 *C. albicans* gene were up-regulated and 344 genes were down-regulated in responses to at least one of the glucose concentration examined. There are 170 of these genes were up-regulated and 180 genes were down-regulated by 0.01% glucose, indicating about half of glucose-regulated genes are responsive to low glucose levels [42]. Therefore, it is concluded that *C. albicans* and *S. cerevisiae* is acquisitively sensitive to glucose. Hence, at the start of this study, an aim was to confirm the impact of galactose and fructose upon specific mRNAs that encode enzymes required for the assimilation of alternative carbon sources. The transcripts encoding the glyoxylate cycle enzyme isocitrate lyase (Ca*ICL1*), fructose-1,6-biphosphatase (Ca*FBP1*), malate synthase (Ca*MLS1*) and multifunctional enzyme of the peroxisomal fatty acid beta-oxidation pathway (Ca*FOX2*) were the main focus here.

C. albicans cells were grown to mid-exponential phase in media containing lactate or oleic acid as the sole carbon source using the same procedures [35]. Galactose or fructose was then added to a final concentration of 2%, samples taken for RNA analysis at various times thereafter, and the levels of the Ca*ICL1*, Ca*FOX2*, Ca*MLS1*, Ca*FBP1* and Ca*ACT1* mRNAs were measured. The relative expression of Ca*ICL1*, Ca*FBP1*, Ca*FOX2* and Ca*MLS1* (compared to the internal Ca*ACT1* control) was high in lactate- and oleic acid-grown cells compared to cells that were exposed to either galactose or fructose.

3. Role of ubiquitination in sugar phosphate-accelerated protein degradation in *C. albicans*

Research done by Sandai et al. [35] suggested that *S. cerevisiae* has retained the ability to degrade target proteins in response to glucose, but that *C. albicans* isocitrate lyase (*ICL1*) has

(A) *C. albicans* and *S. cerevisae* Fbp1

```
CaFbp1    MSGPVNSVSK--QMNVDTDIITLTRFILQEQQTVAPTATGELSLLLNALQFAFKFIAHNI 58
ScFbp1    MPTLVNGPRRDSTEGFDTDIITLPRFIIEHQKQFK-NATGDFTLVLNALQFAFKFVSHTI 59

CaFbp1    RRAELVNLIGVSGSANSTGDVQKKLDVIGDEIFINAMRSSNNVKVLVSEEQEDLIVFPG- 117
ScFbp1    RRAELVNLVGLAGASNFTGDQQKKLDVLGDEIFINAMRASGIIKVLVSEEQEDLIVFPTN 119

CaFbp1    GGTYAVCTDPIDGSSNIDAGVSVGTIFGVYKLQEGSTGGISDVLRPGKEMVAAGYTMYGA 177
ScFbp1    TGSYAVCCDPIDGSSNLDAGVSVGTIASIFRLLPDSSGTINDVLRCGKEMVAACYAMYGS 179

CaFbp1    SAHLALTTGHGVNLFTLDTQLGEFILTHPNLKLPDTKNIYSLNEGYSNKFPEYVQDYLKD 237
ScFbp1    STHLVLTLGDGVDGFTLDTNLGEFILTHPNLRIPPQKAIYSINEGNTLYWNETIRTFIEK 239

CaFbp1    IKK-------EGYSLRYIGSMVADVHRTLLYGGIFAY------PTLKLRVLYECFPMALL 284
ScFbp1    VKQPQADNNNKPFSARYVGSMVADVHRTFLYGGLFAYPCDKKSPNGKLRLLYEAFPMAFL 299

CaFbp1    MEQAGGSAVTIKGERILDILPKGIHDKSSIVLGSKGEVEKYLKHVPK-- 331
ScFbp1    MEQAGGKAVNDRGERILDLVPSHIHDKSSIWLGSSGEIDKFLDHIGKSQ 348
```

(B) *C. albicans* and *S. cerevisae* Fox2

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CaFox2    MS-PIDFKDKVVIITGAGGGLGKYYSLEFAKLGAKVVVNDLGGALDGQGGNSKAADIVVD 59
ScFox2    MPGNLSFKDRVVVITGAGGGLGKVYALAYASRGAKVVVNDLGGTLGGSGHNSKAADLVVD 60

CaFox2    EITKNGGVAVADYNNVLD-GAKIVETAVKSFGTVHIIINNAGILRDSSIKKMTEKDFKLV 118
ScFox2    EIKKAGGIAVANYDSVNENGEKIIETAIKEFGRVDVLINNAGILRDVSFAKMTEREFASV 120

CaFox2    IDVHLNGAYAVTKAAWPYFQKQKFGRVVNTSSPAGLYGNFGQTNYSAAKSALLGFAETLA 178
ScFox2    VDVHLTGGYKLSRAAWPYMRSQKFGRIINTASPAGLFGNFGQANYSAAKMGLVGLAETLA 180

CaFox2    KEGDRYNIKANAIAPLARSRMTESILPPPILEKLGPEKVAPLVLYLSSAENEVTGQFFEV 238
ScFox2    KEGAKYNINVNSIAPLARSRMTENVLPPHILKQLGPEKIVPLVLYLTHESTKVSNSIFEL 240

CaFox2    AAGFYAQIRWERSGGVLFKPD-QSFTAEVVAKRFSEVLNFDDSGKPEYLKNQHPFMLNDY 297
ScFox2    AAGFFGQLRWERSSGQIFNPDPKTYTPEAILNKWKEITDYRD--KP-FNKTQHPYQLSDY 297

CaFox2    TTLTTEARKLPSNDASGAPKVTLKDKVVLITGAGAGLGKEYAKWFARYGAKVVVNDFKDA 357
ScFox2    NDLITKAKKLPPNEQGSVKIKSLCNKVVVVTGAGGGLGKSHAIWFARYGAKVVVNDIKDP 357

CaFox2    TKTVEEIKAAG--GEAWADQHDVASQAEEIIKNVIDKYGTIDVLVNNAGILRDKSFAKMS 415
ScFox2    FSVVEEINKLYGEGTAIPDSHDVVTEAPLIIQTAISKFQRVDILVNNAGILRDKSFLKMK 417

CaFox2    DQEWDQVQKVHLLGTFNLSRLAWPYFAEKKYGRIVNISSTSGIYGNFGQANYASAKAGIL 475
ScFox2    DEEWFAVLKVHLFSTFSLSKAVWPIFTKQKSGFIINTTSTSGIYGNFGQANYAAAKAAIL 477
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CaFox2    GLSKTLAVEGARNNIKVNVVAPHAETAMTLTIFREQDK-NLYHADQVAPLLVYLGSEEVE 534
ScFox2    GFSKTIALEGAKRGIIVNVIAPHAETAMTKTIFSEKELSNHFDASQVSPLVVLLASEELQ 537

CaFox2    ------VTGETFEAGGGWIGNTRWQRAKGAVSHDEHTTVEFIRDNLKDITNFDSDTENPK 588
ScFox2    KYSGRRVIGQLFEVGGGWCGQTRWQRSSGYVSIKETIEPEEIKENWNHITDFSRNTINPS 597

CaFox2    STTESSMAILSAVGGDDDDDDDEEEEDEGDEEEEEEDEEEDDPVWRFNDRDVILYNIALG 648
ScFox2    STEESSMATLQAV----------------QKAHSSKELDDGLFKYTTKDCILYNLGLG 639

CaFox2    ATTKQLHYVYENDSDFQVIPTFGHLITFNSGKSQNSFAKLLRNFNPMLLLHGEHYLKVHK 708
ScFox2    CTSKELKYTYENDPDFQVLPTF-AVIPFMQATATLAMDNLVDNFNYAMLLHGEQYFKLCT 698

CaFox2    WPPPTEGAIKTTFEPISTTPK-GSNVVIVHGSKSVDNDSGEVIYSNEATYFIR--NCQAD 765
ScFox2    PTMPSNGTLKTLAKPLQVLDKNGKAALVVGGFETYDIKTKKLIAYNEGSFFIRGAHVPPE 758

CaFox2    NKVYAERRS-FATNPFPAPK-RAPDYQVDVPISEDLAALYRLTGDRNPLHIDPNFAKGAK 823
ScFox2    KEVRDGKRAKFAVQNFEVPHGKVPDFEAEISTNKDQAALYRLSGDFNPLHIDPTLAKAVK 818

CaFox2    FPKPILHGMCTYGLSAKVLIDKFGMFDEIKARFTGIVFPGETLRVLAWKESDDTVVFQTH 883
ScFox2    FPTPILHGLCTLGISAKALFEHYGPYEELKVRFTNVVFPGDTLKVKAWKQGS-VVVFQTI 877

CaFox2    VVDRGTIAINNAAIKLVGDKAKI 906
ScFox2    DTTRNVIVLDNAAVKLSQAKSKL 900
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(C) *C. albicans* and *S. cerevisae* Icl1

```
CaIcl1    MPY----TPIDIQKEEADFQKEVAEIKKWWSEPRWRKTKRIYSAEDIAKKRGTLK-INHP 55
ScIcl1    MPIPVGNTKNDFAALQAKLDADAAEIEKWWSDSRWSKTKRNYSARDIAVRRGTFPPIEYP 60

CaIcl1    SSQQADKLFKLLEKHDADKTVSFTFGALDPIHVAQMAKYLDSIYVSGWQCSSTASTSNEP 115
ScIcl1    SSVMARKLFKVLEKHHNEGTVSKTFGALDPVQISQMAKYLDTIYISGWQCSSTASTSNEP 120

CaIcl1    SPDLADYPMDTVPNKVEHLWFAQLFHDRKQREER-LTLSKEERAKT-PYIDFLRPIIADA 173
ScIcl1    GPDLADYPMDTVPNKVEHLFKAQLFHDRKQLEARSKAKSQEELDEMGAPIDYLTPIVADA 180

CaIcl1    DTGHGGITAIIKLTKMFIERGAAGIHIEDQAPGTKKCGHMAGKVLVPVQEHINRLVAIRA 233
ScIcl1    DAGHGGLTAVFKLTKMFIERGAAGIHMEDQTSTNKKCGHMAGRCVIPVQEHVNRLVTIRM 240

CaIcl1    SADIFGSNLLAVARTDSEAATLITSTIDHRDHYFIIGATNPEAGDLAALMAEAESKGIYG 293
ScIcl1    CADIMHSDLIVVARTDSEAATLISSTIDTRDHYFIVGATNPNIEPFAEVLNDAIMSGASG 300

CaIcl1    NELAAIESEWTKKAGLKLFHEAVIDEIKNGNYSNKDALIKKFTDKVNPLSHTSHKEAKKL 353
ScIcl1    QELADIEQKWCRDAGLKLFHEAVIDEIERSALSNKQELIKKFTSKVGPLTETSHREAKKL 360

CaIcl1    AKELTGKDIYFNWDVARAREGYYRYQGGTQCAVMRGRAFAPYADLIWMESALPDYAQAKE 413
ScIcl1    AKEILGHEIFFDWELPRVREGLYRYRGGTQCSIMRARAFAPYADLVWMESNYPDFQQAKE 420
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CaIcl1    FADGVKAAVPDQWLAYNLSPSFNWNKAMPADEQETYIKRLGKLGYVWQFITLAGLHTTAL 473
ScIcl1    FAEGVKEKFPDQWLAYNLSPSFNWPKAMSVDEQHTFIQRLGDLGYIWQFITLAGLHTNAL 480

CaIcl1    AVDDFSNQYSQIGMKAYGQTVQQPEIEKGVEVVKHQKWSGATYIDGLLKMVSGGVTSTAA 533
ScIcl1    AVHNFSRDFAKDGMKAYAQNVQQREMDDGVDVLKHQKWSGAEYIDGLLKLAQGGVSATAA 540

CaIcl1      MGQGVTEDQFKESKAKA 550
ScIcl1      MGTGVTEDQFKENGVKK 557
```

Legend:

Label	Score range	Sensitivity	Specificity
Low confidence	0.62 ≤ s ≤ 0.69	0.464	0.903
Medium confidence	0.69 ≤ s ≤ 0.84	0.346	0.950
High confidence	0.84 ≤ s ≤ 1.00	0.197	0.989

(D)

Strain	Number of high confident ubiquitination site			
	Icl1	Mls1	Fox2	Fbp1
C. albicans	0	0	1	0
S. cerevisae	2	0	0	0

Figure 1. Comparison of an amino acid pile-up of (A) CaMls1 and ScMls1. (B) CaFbp1 and ScFbp1. (C) CaFox2 and ScFox2. (D) CaIcl1 and ScIcl1. In which the high confidence ubiquitination sites are highlighted. (E) Compilation of high ubiquitination site in *C. albicans* and *S. cerevisiae* Icl1, Mls1, Fox2 and Fbp1.

lost the specific signal(s) that trigger this glucose-accelerated protein degradation. However, studies have not been done on the effect of other sugar phosphate such as galactose or fructose, both are important carbon sources that are absorbed directly into the blood from intestine. This studies focus on whether CaIcl1 protein has lost the specific signal that trigger protein degradation with the formation of ubiquitin-proteasome complex.

Ubiquitination play an important role in the glucose-accelerated degradation of gluconeogenic enzymes in *S. cerevisiae* [20] by acting as a degradation signal in *S. cerevisiae* [44]. This fact is supported by Eschrich et al. [22], in which ubc8 functions in the catabolite degradation of fructose-1,6-biphosphatase in *S. cerevisiae*. Therefore, consensus ubiquitination target sites were examined in CaIcl1 and ScIcl1, CaMls1 and ScMls1, CaFox2 and ScFox2, and CaFbp1 and ScFbp1 using ubpred (predictor of protein ubiquitination site, from http://www.ubpred.org/index.html) [45, 46] (**Figure 1**).

Based on this bioinformatics comparison, the ScIcl sequence containing strong consensus ubiquitination sites at amino acid 158 and 551, but there is a lack of high confidence ubiquitin target in CaIcl1, CaMls1, ScMls1, CaFbp1 and ScFbp1 (**Figure 1**). Interestingly, CaFox2 contain one strong consensus ubiquitination site at amino acid 588, but there is none high confidence ubiquitination target in ScFox2. This prediction was based on high level of confidence which is described in UbPred system containing score range $0.84 \leq s \leq 1.00$, 0.197 for sensitivity and 0.989 for specificity. However, focus of this study is on Icl1 because it is more important for *C. albicans* virulency. Deletion of *ICL1* gene in *C. albicans* attenuated virulence [27] while deletion of FOX2 confers mild attenuation of virulence of *C. albicans* [26].

The bioinformatics screening of ubiquitination site includes the hydrophobic nature of the ubiquitination target site for high confidence prediction (TEDQFKENGVKK), which is contrast to the low and medium confidence sites which contain acidic and basic residues in the putative ubiquitination site (NGVKK; FNWPKAMSVD) [45, 46]. Therefore, the presence of consensus ubiquitination sites in these proteins correlated with glucose-accelerated degradation [35].

4. Overview of carbon sources attribute to the pathogenicity to *C. albicans*

The effect of galactose or fructose on the expression levels of the CaIcl protein in *C. albicans* and to compare this response with the corresponding situation in *S. cerevisiae* were tested. The effects of glucose on *S. cerevisiae* fructose-1,6-bisphosphatase (FBPase) have been intensively studied and it was reported that the FBPase protein (ScFbp1) is rapidly degraded upon addition of glucose [47]. It is also reported by Hammerle et al. [48] that the levels of cytosolic malate dehydrogenase, fructose-1,6-biphosphatase, isocitrate lyase and phosphoenolpyruvate carboxykinase are all low in *S. cerevisiae* after glucose addition. However, the degradation of FBPase is not correlated with the prediction of ubiquitination target site presence in the protein as ScFbp1 has only one low confidence ubiquitination target site at amino acid 250 (**Figure 1B**). The reason is not known whether or not FBPase had undergone ubiquitin-proteasome machinery although Regelmann et al. [49] have found that there are two different degradation pathways: cytosolic ubiquitin-proteasome machinery, and the other dependent on vacuolar proteolysis. Genes that are essential for vacuolar degradation are unnecessary for proteasome-dependent degradation [49].

Therefore, as a starting point whether Icl1 decline in *S. cerevisiae* upon galactose or fructose addition. To achieve this, the *S. cerevisiae* which is tagged at its 3′-end with Myc_9 is obtained from Sandai et al. [35]. Cells were grown on lactate, and then subjected to 2% of galactose or fructose. Samples were harvested at various times point and the Icl1 proteins expression was determined by western blotting and normalized by beta Actin. Clearly galactose or fructose addition led to the degradation of ScIcl1. These results confirmed that in *S. cerevisiae*, Icl1 are degraded in response to galactose or fructose. Similar results are observed in study done by Sandai et al. [35], whereby ScIcl1 and ScPck1 are degraded in response to glucose.

Next was to determine the effect of galactose or fructose addition to *C. albicans* cell growing on lactate. *C. albicans* strains expressing Myc_3-tagged Icl1 was confirmed by western blotting. *C. albicans* cells were grown in media containing the non-fermentable carbon sources lactate as sole carbon sources and the 2% galactose or fructose was added while cells were in exponential phase. Cells were then harvested at various times, and their proteins extracted and were loaded equally onto the SDS-PAGE gels, and expression of the Myc_3-tagged CaIcl1 protein is detected with anti-Myc antibodies and normalized by beta-actin. The *C. albicans* Icl1 protein was expressed during growth on lactate and was not destabilized by the addition of 2% galactose. Indeed CaIcl1 proteins levels were not significantly different from the control (lactate) even after 4 hours. Likewise, similar result was obtained by Sandai et al. [35] in which

CaIcl1 proteins remained stable after addition of 2% glucose to lactate grown cells. However, interestingly CaIcl1 protein is destabilized after addition of 2% fructose to lactate grown cells. This result contradicts the finding that CaIcl1 has lost the specific signal(s) that trigger protein degradation. This might prove the points by Regelmann et al. [49] that except from ubiquitin proteasome pathways the protein can undergo degradation through vacuolar proteolysis. However, the exact mechanism of why fructose triggers such pathway is not known.

Testing was carried out to see whether *C. albicans* is able to degrade proteins in response to galactose or fructose, the *S. cerevisiae ICL1* gene is expressed in *C. albicans*. *C. albicans ICL1* allele was replaced with a tagged *S. cerevisiae ICL1*. Ca(ScIcl1-Myc_3) was then grown in lactate before subjected to 2% galactose or fructose. Western blot was performed to confirm the expression of the Myc_3-tagged ScIcl1 which will be normalized to housekeeping protein beta-actin. From the results, ScIcl1 protein expression remained stable in *C. albicans* cells grown on lactate. However, following addition of 2% galactose or fructose to *C. albicans* cells, ScIcl1 was degraded. This indicates that *C. albicans* has retained capacity to destabilize target proteins in response to galactose or fructose. In other words, ScIcl1 retained its molecular apparatus for protein degradation even if it is expressed in *C. albicans* cells.

Testing was also carried out to determine whether *C. albicans* Icl1 expression remains stable in response to galactose or fructose, the *C. albicans ICL1* gene was expressed in *S. cerevisiae*. *S. cerevisiae ICL1* allele was replaced with a tagged *C. albicans ICL1.* Sc(CaIcl1-Myc_3) was then grown in lactate before subjected to 2% galactose or fructose. Cells were harvested at different time points thereafter and western blot was performed to confirm the expression of the Myc_3-tagged CaIcl1 which is normalized to beta-actin. From the results, CaIcl1 protein expression remained stable in *S. cerevisiae* cells grown on lactate. However, following addition of 2% fructose to *S. cerevisiae* cells, CaIcl1 was degraded. This supports the previous result that *C. albicans* Icl1 although lacks ubiquitination site but is sensitive to fructose and might undergo a different proteolysis. *C. albicans* has lost capacity to destabilize target proteins in response to 2% galactose. In other words, CaIcl1 was not degraded and remained stable in *S. cerevisiae*.

S. cerevisiae Icl1 has two high confidence putative ubiquitination sites located at residues 551 and 158, whereas CaIcl1 has no such sites (**Figure 1**). Therefore, ubiquitination plays a role in glucose-accelerated protein decay in *C. albicans*; the addition of a ubiquitination site to CaIcl1 would confer glucose-accelerated degradation upon this protein Sandai et al. [35]. Therefore carboxy-terminal ubiquitin site from ScIcl (TEDQFKENGVKK) was added into CaIcl1, together with the Myc_3 tag into the wild type polyubiquitin containing *C. albicans* cells. The function of ubiquitination in regulation rewiring of *C. albicans* Icl1 in response to galactose or fructose was tested. Cells were grown in lactate and galactose or fructose was added to final concentration of 2%. Cells were harvested at different time points thereafter, protein extracted and quantified, and these subjected to western blotting. The CaIcl-Ubi-Myc_3 proteins are normalized to housekeeping protein beta-actin. The result obtained shows that CaIcl-Ubi-Myc_3 is degraded upon addition of galactose or fructose to non-fermentable carbon sources, lactate. The results confirm a finding in a research done by Sandai et al. [35] whereby additions of glucose to lactate grown DSCO4 cells degrade the protein. This has also proven that ubiquitination plays a significant role in regulation of central metabolism enzymes in both *C. albicans* and *S. cerevisiae*.

5. Conclusion and future perspective

The assimilation of carbon sources is fundamentally important for the growth of *C. albicans* and for the establishment of infections in the human host [10]. For most yeast, such as *S. cerevisiae*, glucose is generally a more favorable carbon sources and the chosen mode of metabolism is often fermentative via glycolytic pathway-Embden-Meyerhof fermentation. In the presence of excess glucose, *S. cerevisiae* utilize glycolytic pathways and produce ethanol. *S. cerevisiae* is Crab-negative yeast that has the ability to produce ethanol even in the absence of oxygen [50]. In contrast, *C. albicans* is Crab-positive yeast because it retains respiratory capacity in the presence of excess glucose [51].

C. albicans is glucose Crabtree positive yeast which retains respiratory activity even following exposure to glucose [51]. During growth on glucose, *ADH1* mRNA levels rise to maximum levels during late exponential growth phase and the decline to low levels in stationary phase [52]. The *ADH1* mRNA is relatively abundant during growth on galactose, glycerol, pyruvate, lactate or succinate, and less abundant during growth on glucose or ethanol. However, alcohol dehydrogenase levels do not correlate closely with *ADH1* mRNA levels. This locus may be controlled in both transcriptional and post-transcriptional levels, or other differentially regulated *ADH* loci may exist in *C. albicans* [51]. Interestingly, a significantly smaller proportion of glucose is fermented to ethanol by *C. albicans* than by *S. cerevisiae* [51].

S. cerevisiae is not able to assimilate both non-fermentable carbon sources and galactose/fructose at the same time because of repression. Hence, these yeasts have evolved different responses to galactose/fructose. Therefore in this analysis was done on regulation of carbon assimilation in *C. albicans* focusing on genes/enzymes involved in gluconeogenesis, glyoxylate cycle and β-oxidation. *ICL1*, *FOX2*, *MLS1*, and *FBP1* gene expression and the impact of galactose or fructose on the assimilation of non-fermentable carbon sources were analyzed. *C. albicans* responses were then compared to those of *S. cerevisiae* under equivalent conditions. The following conclusions can be drawn from these findings;

First, gluconeogenic, glyoxylate cycle and β-oxidation mRNAs are sensitive to galactose or fructose in both *C. albicans* and *S. cerevisiae*. This reconfirmed previous finding, whereby the transcriptome, such as *PCK1* and *FBP1* in *S. cerevisiae* [42], *ICL1* and *PCK1* in both *S. cerevisiae* and *C. albicans* [35], was degraded upon exposure to glucose. The dramatic decreases in *ICL*, *MLS1*, *FOX2* and *FBP1* mRNA levels in *C. albicans* after exposure to 2% galactose or fructose to lactate- and oleic acid-grown cells was observed. It is already known that *C. albicans* sensitive to glucose concentrations even as low as 0.01%, this value is within the physiological range of blood glucose (about 0.1%) [10, 12]. Therefore, *C. albicans* is able to respond to blood glucose levels during disseminated hematological infections. Interestingly, diabetic patients who often have elevated blood glucose levels have a higher risk of systemic *Candida* infections [53] and dietary glucose enhances *C. albicans* colonization and invasion [54]. Galactose and fructose are both important sugar phosphate to human and yeast. Galactose can be converted to glucose through Leloir pathway which utilized three enzymes in human liver [55] and five enzymes in yeast [56]. In mammary gland galactose covalently bound to glucose to formed disaccharide lactose [57]. Moreover individual with galactosemia, a rare inherited metabolic disorder that affects the body ability to metabolize galactose properly and have high content

of galactose in both urine and blood. This increases the risk of developing *E. coli* sepsis and sometimes or rarely fungal sepsis, such as *Candida* species [58].

Second main observation was that the Icl1 proteins are stable in *C. albicans* following galactose exposure but not fructose. The addition of 2% galactose to *C. albicans* cells growing on lactate did not trigger the degradation of the Icl1 proteins, but the protein was degraded upon addition of 2% fructose, at least 4 hours examined. This is in contrast to the situation in *S. cerevisiae*, where the addition of 2% galactose or fructose triggered the rapid degradation of the Icl1 proteins. The estimated half-lives for these proteins in *S. cerevisiae* are more than 20 hours [46] indicating that these proteins are very stable. This probably represents a significant difference in the physiological responses of these pathogenic and benign yeasts to glucose. *C. albicans* is able to establish infections in complex niches, many which contain a rich mixture of alternative carbon sources [10]. The stability of the Icl1 protein in *C. albicans*, even in the presence of galactose and glucose [35] might suggest that this pathogen is capable to assimilate alternative carbon sources at the same time as galactose or glucose in these carbon-rich niches. The observation from addition of galactose and glucose is similar in *C. albicans*, this may be because galactose and glucose is reversibly convertible in Leloir pathway, and galactose is first converted to glucose to enter the glycolysis pathway.

Interestingly there is a finding that fructose increases phosphorylation/activation of hypothalamic AMP kinase causing phosphorylation/inactivation of Acetyl-CoA carboxylase, whereas glucose has the inverse effects [59]. This finding is interesting because acetyl-coenzyme A (Acetyl-CoA) is an essential cofactor in central metabolism, this molecule is the entry point to the tricarboxylic acid (TCA) cycle that generates energy, biomass, and intermediates for macromolecules [59]. This means that addition of fructose might repressed Acetyl-CoA in *C. albicans* which will in turn affects the TCA and glyoxylate cycle. However, this might not be the only reason for Icl1 protein decay in *C. albicans* in response to fructose. Glucose enters the glycolytic pathway via glucokinase-catalyzed phosphorylation and its further metabolism is subjected to control in phosphofructokinase (PFK) in the glycolytic pathway, which is considered to be rate-limiting step. In contrast, fructose is metabolized faster than glucose because it bypasses this step entering the glycolytic pathway at the level of the triose phosphates. This showed that fructose is a much efficient carbon sources compared to glucose or alternative non fermentable carbon sources such as lactate.

The third observation whether *C. albicans* has lost signal that trigger destabilization in response to galactose and retained molecular capability of destabilizing target proteins in response to fructose. *C. albicans ICL1* ORF was expressed in *S. cerevisiae* and vice versa *S. cerevisiae ICL1* ORF was expressed in *C. albicans*. The CaIcl1 in *S. cerevisiae* remained stable even after addition of 2% galactose but was degraded after addition of 2% fructose was tested. The finding supported the previous report. This is interesting because it proved that *C. albicans* Icl1 is sensitive to fructose but not to galactose. In contrast, ScIcl1 in *C. albicans* was degraded in response of 2% galactose or fructose.

The analysis of ubiquitination in *C. albicans* was done by exposing CaIcl1-Ubi-Myc_3 to 2% galactose or fructose. *C. albicans* Icl1 is destabilized by galactose because of the addition of carboxyl-terminal ubiquitination site. In previous finding the fructose destabilize CaIcl1 and addition of ubiquitin site accelerated the destabilization of the protein. Cells

can no longer able to metabolize via the glyoxylate cycle following galactose and fructose addition due to the degradation of Icl1 protein. As a result, those cells would presumably be less able to cause infections and would be less able to compete for available nutrients against other microorganisms such as endogenous bacteria in the gastrointestinal (GI) tract compared to their wild type. It is likely to show less successful colonization, virulence and fitness due to this defect in its ability to assimilate both glucose and alternative carbon sources at the same time. Similarly, mutant *C. albicans* cell with deletion of *ICL1* attenuated virulence [27].

It is interesting to further study the mechanism involved in protein degradation in response to fructose. Why is only fructose and not glucose or galactose that triggers protein degradation of CaIcl1? And which pathways could CaIcl1 take for proteolysis? Is it vacuolar proteolysis or ubiquitin mediated proteolysis, such as ubiquitin conjugating enzyme Ubc8p [43] and E3 ubiquitin ligase, Gid complex [49, 60]? It is also interesting to explore other central metabolism enzymes that carry the same characteristic of CaIcl1 and also to determine the effect of exposure to other carbon sources such as malate and succinate.

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Chapter 4

The Cell Wall of *Candida albicans*: A Proteomics View

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Abstract

Candida species are a natural commensal of humans and when the immune system is compromised it can cause candidiasis. *C. albicans* is the main etiological agent of candidiasis, representing nearly 60% of the total cases worldwide. The cell wall provides protection against several physical and chemical aggressions and is responsible for the different shapes displayed by *C. albicans*. The cell wall is not a static structure, but a dynamic one, having great plasticity to allow different cell morphologies, molecular remodeling and changes in the cell wall composition as a result from adaptation to the surrounding environment. It is mainly composed of chitin, β-glucan and mannoproteins. Therefore, the cell wall components are putative targets for the discovery and development of new drugs. The cell wall reprograming in response to several conditions, including a host carbon source, blood, serum, high temperature, acidic environment, and morphogenesis, have a direct impact on the mannoprotein content and might be involved in adherence, drug resistance and virulence of *C. albicans*. In this chapter, we performed an analysis of the proteins that have been identified in the *C. albicans* cell wall by our group and others, which allowed the identification of proteins from different intracellular compartments.

Keywords: *Candida albicans*, cell wall, proteomics, review

1. Introduction

Fungal infections are a serious health concern worldwide. There has been estimated that more than 13% of the total human population has a fungal infection on skin, 300 million people are suffering a severe fungal infection, and 25 million are in great danger of dying or blindness as a

result of an infection by fungi [1]. Infections caused by fungi provoke over 1.5 million deceases worldwide, mainly in people carrying HIV infections or suffering two or more pathological conditions [2]. Even though there are more than 150 species of *Candida*, only nearly 20 species are known to cause human infections, being *C. albicans* the most frequent causative agent of candidiasis and the leading fungal infection [3]. *C. albicans* is part of the normal microflora in humans and can be found in normal mucosae or cutaneous microflora of healthy individuals [2]. Under certain conditions, as when patients are being treated with antibiotics, immunosuppressive drugs, or chemotherapeutic agents, or are in surgical intensive care units for prolonged time, the commensal *C. albicans* yeast cells turn into pathogenic cells implicated in life-threatening invasive candidiasis [3]. Moreover, there have been increasing reports of *C. albicans* strains exhibiting a multidrug resistance phenotype to antifungal drugs. Therefore, *Candida* infection is a clinical problem worldwide due to the difficulty of treating systemic candidiasis.

2. The cell wall of *Candida albicans*

C. albicans has developed several molecular tools for sensing and evading the host immune system, which include fungal virulence factors involved in adherence, cell damage, and invasion [4]. Many of these factors are localized in the fungal cell wall, which constitute the main structure to be in contact with host cells and is essential for the fungal cell integrity. The cell wall is a remarkable structure because of its key role in protection against environmental stress conditions, including osmotic changes, dehydration, heat, cold, immune system response, or attack by other microorganisms [5–7], and it is also responsible for the cell shape and adhesion to host cells through adhesins.

The cell wall is mainly composed of proteins, glycans, and chitin [5–7]. Cell wall proteins are heavily mannosylated through *O*- and *N*-chemical bonds and function as structural elements, adhesins, cross-linking enzymes, molecular sensors of environmental changes, as well as in protection from environmental changes [5–8]. There are two classes of covalently bound fungal cell wall proteins (CWPs), the glycosylphosphatidylinositol (GPI)-dependent CWPs and Pir-CWPs [5–7], which can be isolated from cell walls by treatment with mild alkali (alkali-sensitive linkage, ASL). In the case of *Saccharomyces cerevisiae*, Cwp2 is a GPI cell wall protein harboring a Pir repeat involved in linking Cwp2 to β-1,3-glycan for increasing cell wall integrity [6, 8]. Proteins lacking homology to Pir proteins have been designated alkali-sensitive linkage cell wall proteins (ASL-CWPs), and are covalently linked to the cell walls of *C. albicans* and *S. cerevisiae* by mild-alkali sensitive chemical bonds [5, 6, 8]. Other proteins are linked to CWPs through disulfide bonds [9].

Most of the cell wall components have covalent linkages established to enhance the cell wall stability and, consequently, protection against environmental changes. One type of proteins described as main cross linkers of cell wall components are the members of the gas family that only contains members of the fungal kingdom. *C. albicans* possesses five genes encoding members of this family, the *PHR1*, *PHR2*, *PHR3*, *PGA4*, and *PGA5* genes [10]. These proteins are β-1,3-glucanases that also exhibit transglucosidase activity and are needed for cross linking β-1,3-glucan and

β-1,6-glucan. *PHR1* and *PHR2* genes are the only members of the family that are responsive to changes in pH [10]. *PHR1* gene is able to complement the *S. cerevisiae GAS1/GGP1* null mutant, which has as main phenotypic characteristics, spherical shape, and affectation of normal budding pattern in the stationary phase of growth curve, indicating that the *PHR1* gene is the ortholog of *GAS1/GGP1* in *C. albicans* [11]. *PHR2* gene encodes a protein that shows 55.15 and 34.48% identity to Phr1 and Phr3, respectively, while Phr1 has 32.03% identity to Phr3, and interestingly, Phr3 does not have a GPI-anchoring site. *PGA4* and *PGA5* genes, the other members of the Gas family, encode proteins that share 25–35% identity to Phr1 and Phr2, and are not pH responsive.

Other proteins involved in the formation of covalent linkages are transglutaminases (TGases), a group of enzymes that are widely distributed in microorganisms, plants, and animals. They have been involved in a number of biological processes, such as differentiation, growth regulation, cellular adhesion, and tissue integrity. These proteins are enzymes with several functions, including posttranslational modifications, protein cross linking, incorporation of amine groups, and deamination. The best studied TGase function is cross linking, which involves a transamination reaction between the side chains of glutamine and lysine residues in target proteins to form N-ε-(γ-glutamyl) lysine amide bonds, rendering an increase in mechanical strength and resistance to proteolytic degradation [12]. Most of the transglutaminases are fully dependent of Ca^{2+} ions for their activity. However, there are transglutaminases from microorganisms that do not require Ca^{2+} for their function. Similarly, TGases from rodent intestinal mucosa are also independent of Ca^{2+} for their enzymatic activity [12, 13]. One of the best studied transglutaminases is the human TGase 2 that has a key role in the stabilization of the extracellular matrix and modulation of the interaction between fibronectin and integrin [14]. In addition, it has been involved in several human diseases of great impact in health worldwide, such as Alzheimer's, Huntington, and Crohn's diseases, as well as in fibrosis, cancer and other diseases [15]. In the case of *Chlamydomonas reinhardtii*, TGase has a key role in the assembly of cell wall through the cross linking of the cell wall components [16]. TGase activity has also been reported in *C. albicans* and *S. cerevisiae*, where it was found localized in the cell walls fraction of yeast cells and showed the capacity to establish covalent cross links between proteins. TGase activity from these microorganisms was inhibited by the specific inhibitor cystamine, which diminished the incorporation of several polypeptides into the cell wall, affected the regeneration of protoplasts, and inhibited the yeast-to-mycelium transition, revealing a role in the establishment of covalent cross links between cell wall proteins and the structural polymers chitin and/or glucan [17, 18]. In a recent work, the inhibition of TGase activity with cystamine inhibited the growth of yeast cells and induced autophagy. It also affected the yeast division pattern and the cell wall structure. Interestingly, the protein responsible for TGase activity in cell wall was identified as enolase by mass spectrometry [19].

3. Glucan

Glucan is the main component of the yeast cell wall, and along with chitin, these are assembled into a three-dimensional and dynamic network responsible for cell shape that renders protection against environmental insults. In the dimorphic yeast *C. albicans*, chitin abundance

is approximately 1–2% of dry weight of the cell wall and is located adjacent to the cell membrane. β-1,3 and β-1,6-glucan represent 40% and 20% of dry weight of the cell wall, respectively, and are situated immediately after chitin. The outer layer of the cell wall is mainly composed by mannoproteins that define the cell surface properties [5, 20].

C. albicans glucan is a highly branched polymer. It is composed of linear D-glucose molecules bound through β-1,3- and β-1,6-glycosidic linkages. β-1,3-glucan forms a helical backbone as a single residue or a chain made of three strands attached by hydrogen bonds. β-1,6-glucan is responsible for the ramification due to cross linking with chitin and mannoproteins through a GPI-anchor bonded to the nonreducing end of β-1,3-glucan. Likewise, chitin and Pir proteins are attached to the nonreducing end of the β-1,3-glucan molecule [21]. It has been shown that during biofilm formation, the *C. albicans* glucan increases the resistance to several antifungal drugs, such as amphotericin B, fluconazol [22], and anidulafungin (an echinocandin) because of a mechanism of drug sequestration [23]. Biofilm lifestyle on medical devices is a high challenge on medicine because of the difficulty to treat patients with contaminated prosthetic materials.

The synthesis of β-1,3-glucan involves several steps and enzymes, and the major and best characterized is a plasma membrane complex named glucan synthase that uses UDP-glucose as universal donor and is stimulated by GTP; due to a transglycosylation reaction, the nascent linear chain is extruded into the cell wall where it can be further processed by several enzymes. The complex contains the catalytic subunit Fks/Gsc and the regulatory subunit Rho1 [24]. There has been identified three genes encoding the glucan synthase, *FKS*/*GSC*, *GSC1*, *GSL1* and *GSL2*. The main activity comes from *GSC1* gene and the incapability to obtain the null mutant highlight the importance of this gene [25]. Inhibition of glucan synthesis by blocking Fks1/Gsc1 activity to damage the cell wall is the main target of antifungal drugs. Echinocandins are the most important of these type of drugs. Most of the *C. albicans* clinical isolates are susceptible to these drugs; however, mutations in *FKS* genes result in echinocandin resistance [26], augmented chitin amount [27], and reduced expression of *FKS2* and *FKS3* genes [28]. *FKS2* and *FKS3* null mutants contain an increased amount of wall glucan and are more tolerant to echinocandin drugs indicating that both genes negatively regulate *FKS1* [29].

β-1,3-glucan undergoes some modifications after its synthesis, such as hydrolysis at the nonreducing end or within the glucan molecule, transference and bound to another chain or cross linking to β-1,6-glucan. Xog1/Exg1 is the major β-exoglucanase activity that is secreted to the cell wall and then to the milieu, and has a dual activity as it can also act as a glycosyltransferase [30, 31]. Xog1/Exg1 null mutant has no significant changes in viability, pathogenesis, or morphogenesis compared with the parental strain [32]. Bgl2 (GH17) is an endo-β-glucanase and 1,3-β-glucanosyltransferase in *C. albicans*. The null mutant of this enzyme has more sensitivity to chitin inhibitors and reduced virulence, but glucanosyltransferase activity remains in these null mutants suggesting the presence of other transferases [33]. *BGL2* is up-regulated during cell wall regeneration and when cells are challenged with fluconazole [34]. Interestingly, the absence of exoglucanases and glycosyltransferases augment the sensitivity to antifungal drugs during biofilm formation [22]. Another well-characterized glucanase is Eng1, an endoglucanase member of the family GH81; null mutants form cell chains because mother and daughter cells do not accomplish cytokinesis, indicating Eng1 has a key role in cell division [34].

The synthesis of β-1,6-glucan remains poorly understood. It requires Kre9, an *O*-glycosylated cell surface protein that belongs to the GH16 family. In the *KRE9* null mutant, the amount of β-1,6-glucan is undetectable and GPI-wall proteins are released to the extracellular medium and its virulence is attenuated [35]. Nevertheless, there have identified more genes involved in the synthesis of β-1,6-glucan [5].

4. Chitin

Chitin is a linear polymer of N-acetylglucosamine that corresponds to 1–2% of the dry weight of the yeast cell wall, and reaches up to 10% in hypha [36]. Chitin can also be deacetylated to produce chitosan by the enzymatic action of chitin deacetylases Cda1 and Cda2 [37], which has been reported in the formation of spores in *S. cerevisiae* under conditions of nitrogen starvation and absence of fermentable carbon sources [38]. The synthesis of chitin occurs as a transglycosylation reaction, where N-acetylglucosaminyl residues are transferred from UDP-N-acetylglucosamine (UDP-GlcNAc) to growing polysaccharide chains through β-(1–4) chemical bonds [39]. The *C. albicans* chitin is synthesized by chitin synthases organized into a family of four isoenzymes that are classified into three classes of chitin synthases, class I (Chs2 and Chs8), class II (Chs1), and class IV (Chs3) [39]. These enzymes synthesize chitin, which deposits at the tips during polarized growth of buds and hypha, and in septum. Chs1 protein is required for viability, cell shape, and septum formation. Under conditions of repression, the conditional mutants *Δchs1* can grow, but daughter buds are not separated from mother cells, forming yeast chains [40]. Class I chitin synthases participate in cell integrity during polarized growth in yeasts and hyphae, and contribute to the protection of nascent cell wall during polarized growth, as well as to the integrity of cells under stress affecting the cell wall [41]. Mutant *chs8* yeast cells showed the lack of long chitin microfibrils in septa of yeast and hyphae, while *chs3* mutants showed absence of short microfibrils [42].

The treatment of *C. albicans* with sub-minimum inhibitory concentration (MIC) levels of caspofungins has been reported to cause a compensatory increase in chitin content [43]. Walker et al. [44] showed an increase of chitin content in response to the use of echinocandins in several *Candida* species. The protein kinase C (PKC) and calcineurin signaling pathways were shown to be activated in isolates of *C. albicans*, *C. krusei*, *C. parapsilosis,* and *C. guilliermondii*, generating an increase in chitin and a reduced susceptibility to caspofungin. Thus, this is a mechanism of tolerance to caspofungin in *Candida* species [44].

5. Mannoproteins

The *C. albicans* cell wall mannoproteins correspond to approximately 40% of the cell wall content and are localized in the outermost layer of the cell wall, and consequently, they interact with host proteins and are the first line of defense against host response [45]. One of the main sugar residues in mannoproteins is mannose, which is linked to proteins during the *N*-, *O*-,

and *C*-glycosylation processes in *C. albicans*. The mechanisms leading to these modifications of proteins in *C. albicans* are quite similar to those present in *S. cerevisiae*. There are excellent reviews on this issue in both microorganisms. In this section, we will briefly describe the two main glycosylation processes.

Most of the cell surface glycoproteins are extensively glycosylated and harbor both *N*-linked and *O*-linked oligosaccharides. The *N*-glycosylation of proteins begins with the attachment of the Glc_3Man_9-$GlcNAc_2$ core oligosaccharide by the dolichyl-diphosphooligosaccharide-protein glycosyltransferase complex (OST), where Ost1 is essential for the catalytic activity [46]. This core is attached to the asparagine residue in Asn-X-Ser/Thr sequons of proteins in the lumen of the endoplasmic reticulum, where X is any amino acid but proline. The nature of the X amino acid has a key role in the extent of mannosylation of the outer chain. Hyperglycosylation is inhibited by negatively charged amino acids, while it is enhanced by positively charged residues [47].

The synthesis of the Glc_3Man_9-$GlcNAc_2$ core begins with the synthesis of dolichol-phosphate-mannose (Dol-P-Man) by the dolichyl-phosphate-β-mannosyltransferase or dolichyl-phosphate-mannose synthase (DPMS) using the isoprenoid lipid dolichol-phosphate (Dol-P) and GDP-mannose as substrates, while the dolichol-phosphate-glucose (Dol-P-Glc) is synthesized by the dolichyl-phosphate-β-glucosyltransferase or dolichyl-phosphate-glucose synthase (DPGS or Alg5p) using Dol-P and UDP-glucose as substrates [46]. Both Dol-P-Man and Dol-P-Glc are translocated from the cytosolic face to the lumen of the endoplasmic reticulum by not yet described flippases, where they will be used during the synthesis of the Glc_3Man_9-$GlcNAc_2$ core oligosaccharide. The synthesis of the lipid-linked oligosaccharide Glc_3Man_9-$GlcNAc_2$-P-P-dolichol begins with the transfer of N-acetylglucosamine-phosphate (GlcNAc-P) group to dolichol phosphate (Dol-P) from UDP-GlcNAc by the UDP-N-acetylglucosamine dolichyl-phosphate N-acetylglucosamine phosphotransferase (Alg7) to produce the dolichyl-pyrophosphate-GlcNAc (Dol-PP-GlcNAc), a chemical reaction inhibited by tunicamycin. The second N-acetylglucosamine residue is added by the Alg7, Alg13, and Alg14 protein complex from UDP-GlcNAc to generate Dol-PP-$GlcNAc_2$. The first mannose residue is then added to Dol-PP-$GlcNAc_2$ by the chitobiosyldiphosphodolichol β-(1,4)-mannosyltransferase (Alg1) using GDP-Man, followed by the sequential addition of two mannose residues by the α-1,3/1,6-mannosyltransferase (Alg2p) to generate Dol-PP-$GlcNAc_2Man_3$, being the first mannose linked through an α-1,3 chemical bond and the second through an α-1,6 linkage. These two enzymes are assembled into a protein complex as well. Two mannose residues are then further linked to the α-1,3-mannose of Dol-PP-$GlcNAc_2Man_3$ by the GDP-Man:$Man_3GlcNAc_2$-PP-Dol α-1,2-mannosyltransferase (Alg11) to assemble into Dol-PP-$GlcNAc_2Man_5$. Subsequent reactions occur in the lumen of the endoplasmic reticulum after the Dol-PP-$GlcNAc_2Man_5$ translocation by the oligosaccharide translocation protein (Rft1). The first mannose residue added in the lumen is linked through an α-1,3-chemical bond to the α-1,6-mannose of Dol-PP-$GlcNAc_2Man_5$ by the Dol-P-Man:$Man_5GlcNAc_2$-PP-Dol α-1,3-mannosyltransferase (Alg3). Then, a mannose residue is α-1,2 linked to the α-1,3-mannose of Dol-PP-$GlcNAc_2Man_6$ to generate Dol-PP-$GlcNAc_2Man_7$ by the Dol-P-Man:$Man_5GlcNAc_2$-PP-Dol α-1,3-mannosyltransferase (Alg9). The next mannose residue is linked to the α-1,6-mannose of Dol-PP-$GlcNAc_2Man_7$ by a Dol-P-Man:$Man_7GlcNAc_2$-PP-Dol α-1,6-mannosyltransferase (Alg12), which shows 37% identity and 56% similarity to *S. cerevisiae* Alg12. The last mannose is linked to the α-1,6-mannose of Dol-PP-$GlcNAc_2Man_8$ by the Dol-P-Man:$Man_8GlcNAc_2$-PP-Dol α-1,2-mannosyltransferase (Alg9p) to

get Dol-PP-GlcNAc$_2$Man$_9$. The final steps in the synthesis of the core oligosaccharide are the sequential additions of three glucose residues by the Dol-P-Glc:Man$_9$GlcNAc$_2$-PP-Dol α-1,3-glucosyltransferase (Alg6), Dol-P-Glc:Glc$_1$Man$_9$GlcNAc$_2$-PP-Dol α-1,3-glucosyltransferase (Alg8p) and Dol-P-Glc:Glc$_2$Man$_9$GlcNAc$_2$-PP-Dol α-1,2-glucosyltransferase (Alg10) [46].

The core oligosaccharide attached to proteins is further modified by the three α-glycosidases Cwh41, Rot2, and Mns1 in the endoplasmic reticulum before proteins are transported to Golgi apparatus. The mannosyl-oligosaccharide glucosidase Cwh41 cleaves the outer glucose residue bound through an α-1,2 chemical bond. Rot2 (glucan α-1,3-glucosidase subunit 2) removes the two glucose residues bound through α-1,3 linkages. Finally, Mns1 or mannosyl-oligosaccharide α-1,2-mannosidase removes the mannose residue bound through an α-1,2 linkage rendering the final mature Dol-PP-GlcNAc$_2$Man$_8$ oligosaccharide attached to proteins ready for further modifications in the Golgi apparatus [46].

The synthesis of the mannan outer chain in the Golgi apparatus is initiated by the initiation-specific α-1,6-mannosyltransferase or Och1, which adds a mannose residue through an α-1,6 chemical bond to the inner α-1,3-mannose of the GlcNAc$_2$Man$_8$ core bound to the asparagine residue in proteins. The elongation of the outer chain is first carried out by the mannan polymerase I complex (M Pol I), which adds up to 10 mannose residues trough α-1,6 linkages. M Pol I has several subunits including the mannan polymerase I complex Van1 subunit and the mannan polymerase complex subunit Mnn9. The addition of further mannose residues (up to 50 in yeast) is performed by the α-1,6-mannosyltransferase activity of the mannan polymerase II complex (M Pol II), which contains Mnn9, Anp1, Mnn10, Mnn11, and Hoc1 [48, 49].

After the action of these enzymes, the α-1,6 mannose polymer that constitutes the backbone of the outer mannan chain, is ready for the formation of branches, mainly made of mannose residues linked through α-1,2 chemical bonds, both α-1,2/α-1,3 mannose units, or α-1,2/β-1,2 mannose residues. The addition of the first α-1,2-mannose is performed by the α-1,2-mannosyltransferase Mnn2, and the subsequent α-1,2-mannose residues is carried out by Mnn5 in yeast. *C. albicans* has an ortholog of Mnn2 protein, which shows α-1,2-mannosyltransferase activity and adds the fourth and fifth mannose residues during *O*-glycosylation [50]; however, the mannosylation role during *N*-glycosylation has been assigned to Mnt4 and Mnt5/Ktr2 [50, 51]. The addition of the α-1,3 mannose units is performed by Mnn1 in yeast, but the corresponding enzymatic activity has not yet been identified in *C. albicans*. The addition of β-1,2 mannose residues is performed by Bmt1 and Bmt3 in *C. albicans*. Another modification in oligosaccharide branches is the phosphomannosylation, which occurs in the α-1,2-mannose and is catalyzed by Mnn6 in yeast, while in *C. albicans* is done by Mnt3 and Mnt5/Ktr2. This phosphomannan can also be further modified by the addition of β-1,2 mannose units by the catalytic activity of Bmt2, Bmt3, and Btm4 [50, 51].

The initial step in the *O*-glycosylation process, also known as *O*-mannosylation, is the attachment of an α-1,2 mannose unit to serine or threonine residues on proteins in the endoplasmic reticulum by Pmt1, Pmt2, Pmt4, Pmt5, and Pmt6 enzymes using as substrate Dol-P-Man. The elongation of the *O*-oligosaccharide (up to six mannose units in length) is carried out in the Golgi apparatus. The second and third α-1,2 mannose units are added by Mnt1/Kre2 and Mnt2 enzymes. This *O*-Man$_3$ oligosaccharide can also be modified by the addition of β-1,2

mannose residues by Bmt1 and Bmt3 proteins. The addition of more α-1,2 mannose units is performed by Mnt1 [50, 52].

6. Proteomics of the *Candida albicans* cell wall

C. albicans sequencing genome project has revealed that it possess 6218 open reading frames (ORFs). However, only 1686 ORFs (~27%) have been characterized so far, and 4380 putative OFR's are of unknown function [53]. In order to determine which genes are expressed to proteins, it is necessary to determine the proteome through mass spectrometry, which has become the workhorse in mining proteomes. The cell wall is the first structure to make contact with host, and consequently, several studies have been performed to identify the proteins contained within the cell wall and those involved in the interaction with host human cells. One of the first works to elucidate the high complexity of the *C. albicans* cell wall was carried out by Pitarch et al. [54], who analyzed a series of fractionations from isolated cell walls of either blastospore or hyphae by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Unusual and conventional proteins were identified and grouped on classical CWPs, heat shock and chaperone, folding, elongation factors, glycosylation, fermentation enzymes, and miscellaneous with unknown functions. Proteins highly glycosylated were not successfully identified; nevertheless, identification was possible when PNGase F was used. Likewise, to minimize possible contamination with proteins from different cell compartments, biotinylation has been used to purify CWPs from yeasts and hyphae, prior to cell fractionation [55]. Proteins were purified by affinity chromatography, precipitated, separated by SDS-PAGE, and further identified by (MALDI-TOF-MS). Interestingly, some intracellular proteins were localized in the cell wall during blastospore formation. As an example, the thiol-specific antioxidant-like protein 1 (Tsa1) was found in the nucleus in blastospores, whereas during the hyphae formation, it was localized on the cell surface.

De Groot et al. [56] carried out a series of extractions using isolated cell walls, and the proteins in each fraction were submitted to anion-exchange chromatography. Proteins in fractions were then separated through SDS-PAGE and peptides, after proteinase digestion, were identified with a Quadrupole-TOF (Q-TOF) MS system. Covalently bound CWPs were identified and classified in several protein classes, including carbohydrate-active enzymes, adhesion, host defense, and proteins of unknown function. A total of 12 proteins were identified as GPI-linked proteins and two were classified as sensitive to mild-alkali extraction. In a similar way, peptides from proteins obtained through several chemical extractions of cell walls from blastospores and mycelium, separated through a C18 reverse phase column and identified with an ESI-quadrupole MS system allowed an increased number of identified proteins, both covalently and noncovalently bounded to the cell wall. In the former, most of the proteins were identified as GPI-anchored proteins. In the latter, they were classified in two groups, one named *bona fide* surface proteins, such as agglutinin-like sequence (Als) proteins, glucanases, chitinases, among others, and a group termed atypical cell wall proteins involved in cell cycle, metabolism and energy, protein synthesis, stress response, translation elongation, transport, and others, as well as those with unknown functions that had never been reported

as part of the cell wall. In addition, MS analysis of SDS extracts obtained from cell walls allowed the identification of most of these atypical cell wall proteins and some others, suggesting that it is possible to identify proteins directly form cell wall extracts [57]. Cell surface proteins that were nonbound to the glucan network were identified from *C. albicans* biofilms, blastospores, and hyphae using 2D-fluorescence difference in gel electrophoresis (2D-DIGE) and MALDI-TOF/TOF-MS, finding 108 differentially expressed proteins. Totally, 25 proteins were exclusively found in the biofilm and were classified in different classes, including metabolic process, protein fate and synthesis, cell surface rescue defense and virulence, biogenesis of cellular components, protein metabolism, and uncharacterized [58].

Reference cultures that were metabolically labeled with ^{15}N for relative quantification using an ESI-Fourier transform (FT) ion cyclotron resonance mass spectrometer (ESI FT-ICR MS) have been used to determine the difference among the yeast and hyphal cell wall proteomes, and cultures under different stimulus, including fetal calf serum, N-acetylglucosamine (GlcNAc), and Iscove's modified Dulbecco's medium (IMDM). The latter stimulus showed the strongest hyphal induction [59]. Cell wall fractions were obtained as described in [56] and quantified 21 proteins. Proteins involved in cell wall remodeling and maintenance showed no significant variations in all conditions tested. Several proteins that are specifically linked to hyphal growth were also identified, including Hyr1, Sod5, Hwp2, Plb5 and Als3, as well as to yeasts only, such as Rhd3, Ywp1, and Sod4. Similarly, the effects of fluconazole on the secretome and cell wall proteome and integrity were studied [60], revealing that fluconazole not only affects the cellular membranes, but the cell wall proteome as well, as was shown in both planktonic and sessile cultures, and confirming that fluconazole inhibits the morphogenesis to hyphae, lowering the amount of cell wall proteins distinctive from hyphae, and increasing the number of cell wall repair associated proteins.

Sosinska et al. [61] demonstrated that pH induces modifications of the *C. albicans* cell wall proteome using a system that resembles the mucosal surface. When cells were cultured at pH 7.0, they were mainly characterized by hyphal growth, while at pH 4.0, only yeasts and pseudohyphae were obtained. Relative quantitation of cell wall proteins labeled with ^{15}N using ESI-FT-MS allowed the identification of 21 proteins covalently bounded to the cell wall, being most of them GPI-linked proteins with the exception of Mp65, Pir1, and Tos1. Nine of these proteins were overexpressed at pH 7.0, while 12 were overexpressed at pH 4.0. Additionally, the transglucosidase Phr2 was detected at pH 4.0, while Als1, Als3, Hyr1, Phr1, Rbt1, Sod5, and Tos1 were found at pH 7.0. Moreover, it has been observed that the carbon source provokes changes in the cell wall proteome and secretome [62]. Glucose, lactate, and glucose plus lactate were utilized to induce changes in the cell wall architecture. There was found an upregulation of proteins implicated in cell wall remodeling when cells were grown in lactate in comparison with cells cultured in glucose, modifying the elasticity of the cell wall. Thus, the cell wall structure is modified in response to stress, antifungals, and osmotic changes. In the same way, the cell wall from cells grown as biofilms and lactate, incorporated proteins involved in adhesion, consequently, improving biofilm formation, and adhesion on plastic surfaces.

There has also been shown that incubation of *C. albicans* at 42°C triggers the expression of stress-related proteins [63] as previously described [60], increases both the phosphorylation the Mkc1 MAP kinase (involved in the cell wall integrity) and the chitin amount [64]; on the

contrary, the amount of Cht3 protein is diminished, impeding cell separation after cytokinesis and showing more sensitivity to Calcofluor white, Congo red, and SDS, suggesting that high temperature induces cell wall stress. Mannoproteins covalently linked to the cell wall either by disulfide or alkali-labile chemical bonds were determined after SDS-treatment, deglycosylation, SDS-PAGE, and reverse phase chromatography coupled to an ESI-LTQ-Orbitrap XL MS system [65]. A total of 10 proteins lacking a GPI-anchor were identified; nevertheless, these proteins possess a signal peptide and were classified as cell wall proteins by *in silico* tools. On the other hand, 16 proteins with a GPI-anchor were identified following extraction with β-mercaptoethanol, which were previously identified after HF-pyridine treatment [56]. Moreover, a group of proteins lacking a signal peptide and characteristic of other cell wall compartments, but not from the cell wall, were identified.

Hernández et al. [66] developed an in vitro model to study the yeast-to-hypha transition of *C. albicans* that allowed the determination of the cytoplasmic proteome of hypha that can be used as a reference. This study described the isolation of the cytoplasmic fraction from yeasts and hyphae for 2D-PAGE analysis and spot identification by MALDI-TOF/TOF. There were identified 66 proteins, but only 43 were found involved in diverse metabolic routes; 20 proteins belong to metabolic pathways of amino acids, carbohydrates, nucleotides, lipids and fatty acids, 23 corresponded to enzymes of glycolysis, gluconeogenesis, pentose and tricarboxylic acid pathways, 13 were involved in transcription and protein synthesis, 8 proteins have functions related to cell rescue, virulence and defense, and only 2 with unknown functions.

The cell wall proteome of *C. albicans* ATCC10231 cells cultured with 10% fetal bovine serum (FBS) has been recently published [67]. The morphogenesis induced with FBS at 30 and 37°C revealed that 285 proteins were differentially expressed from a total of 1177. At 30°C, 152 proteins were up-regulated and clustered in several processes, including signal transduction, cell wall biosynthesis, metabolism, stress response, DNA replication, transcription, RNA processing, among others, while 62 were down-regulated and grouped in cell wall proteins, metabolism, stress response, RNA processing, translation and posttranslational modification and transport. At 37°C, 18 proteins were up-regulated and found associated to cell wall biosynthesis, metabolism, translation and posttranslational modifications, and transport, whereas 53 were down-regulated and found with a role in signal transduction, cell wall, metabolism, stress response, transcription, RNA processing, and others. All these data indicate that morphogenesis induced with FBS at 30°C provokes more changes than those observed at 37°C. Thus, FBS induces morphogenesis through several signaling transduction pathways, the inhibition of glycolysis and enhancement of oxidative phosphorylation.

A gel-free method to identify cell surface proteins from alive *C. albicans* cells incubated for 5 min with trypsin and DTT, a technique named cell shaving or surfomics, has been reported [68]. Peptides were separated by RP-HPLC and analyzed in a MALDI-TOF/TOF system. Identified proteins are implicated in cell wall organization and biogenesis, cell rescue, defense and virulence, cytoskeleton organization, transport, metabolism, protein fate and those with unknown function. Using this approach, surfomics differences among yeasts, hyphae and biofilms were determined [69]. A total of 131 proteins covalently and noncovalently bound to the cell wall were identified and 35 of them had never been reported; 27 of these proteins were present in all tested conditions; however, 22 are of unknown functions. Three were found in the biofilm only, 26 on hyphae and 38 on yeast. Proteins were classified in GPI-anchored, cell wall biogenesis

and maintenance, ligand binding, unknown function, and noncell wall function or description. Under this similar method, proteins from planktonic and hyphae cells using an RP-ESI-LTQ-Orbitrap were determined [70]. A total of 943 proteins were found; 423 were shared between yeast and hyphae, 15 were solely detected in yeast and 505 in hyphae. Proteins were classified in cell wall organization and biogenesis, GPI-anchored, cell surface, pathogenesis, stress, plasma membrane, others, and unknown function, being the latter the largest group. About 14 out of 20 of the most abundant proteins were present in both growth styles, 6 were cell surface proteins, 3 involved in stress response, one from pathogenesis, and 4 from other classes. The normalized spectral abundance factor (NSAF) was utilized to calculate the relative protein abundance. Marín et al. [71] challenged *C. albicans* cells with normal human serum (NS) and heat inactivated serum (HIS); they were able to detect human serum proteins covering the cell surface and fungal cell wall proteins. A total of 214 human and 372 *C. albicans* unique proteins were identified, of which 371 proteins were found in NS, 134 in HIS, and 133 proteins were found in both conditions. Hyphae induction with human serum resulted in the identification of 12 proteins that were not found in any other morphogenesis induction. Moreover, 147 out of 372 proteins were classified as cell wall surface proteins, 60 possessed signal peptide, and 23 were GPI-anchored proteins, being glycerol 3-phosphate-dehydrogenase 2 (Gpd2) and pH-regulated antigen 1 (Pra1) the most relevant, both participating in complement evasion, and seven proteins that bind to plasminogen, resulting in immune protection and evasion.

Our group recently performed the ^{14}C-lysine labeling of proteins of the cell wall using them as endogenous substrates by the TGase present in this cellular fraction. Labeled proteins were then sequentially extracted with 2% SDS, and chitinase, separated by 10% SDS-PAGE and the proteins in radioactive areas were identified by tandem mass spectrometry (MS/MS) [19]. Most of the radioactive labeled proteins were identified in the SDS-solubilized fraction (1048 proteins), 37 proteins in the fraction solubilized with zymolyase, and 41 proteins were released with chitinase. Only 24 proteins were extracted in common with the three treatments used. We found the S7A, S13, and S16A 40S ribosomal proteins; 60S ribosomal protein L30; the glycolytic enzymes enolase 1, glyceraldehyde-3-phosphate dehydrogenases 1 and 2, pyruvate kinase, fructose-bisphosphate aldolase, phosphoglycerate kinase 1, phosphoglycerate mutase, and pyruvate decarboxylase; the heat shock proteins of 70 kDa SSA1 and SSB1; the mitochondrial outer membrane protein porin 1 and ADP/ATP carrier protein; the plasma membrane ATPase PMA1, ADP/ATP carrier protein, and galactose transporter-related Hgt7; the elongation factor 1-α 1; the cell wall agglutinin-like protein 1 (Als1) and 2 (Als2), 1,3-β-glucanosyltransferase PGA4, and chitinase 2. About 16 of these proteins have evidence in the cell wall, being Eno1, Pgk1 and Als1 reported as immunodominant proteins [8]. Phosphoglycerate kinase 1 (Pgk1) was shown to confer poor immune protection against *C. albicans* in an infected mouse model [72]. Hsp70 protein is involved in protein folding and translocation, has antigenic properties, and could play a role in the biosynthesis, secretion, and assembly of other components of the cell wall [73]. The full list of cell wall proteins identified from the sequential extractions with 2% SDS, zymolyase, and chitinase has been reported [19].

Asc1, a protein component of the 40S ribosomal subunit and a signal transducer, was found in fractions extracted with both SDS and zymolyase, and has been described as a protein that has a key role in the process of cell-cell adhesion and cell adhesion to substrate. The deletion of *Asc1* showed that this gene could be related to the signaling pathway of the cAMP-dependent protein

kinase Tpk, which has a role in the processes of morphogenesis under differential environmental conditions [61]. Other identified proteins in the SDS cell wall extract were SOD1, SOD2, while SOD5 was found in the extract obtained with zymolyase. These proteins belongs to a family of at least six members in *C. albicans*, whose main function is the transformation of superoxide radicals into hydrogen peroxide that can be subsequently converted into water by catalase enzyme. SOD1 is cytosolic, SOD2 is localized in mitochondria, and SOD5 is a GPI-anchored protein localized in the cell wall [74]. SOD5 has been described as essential for the infection of *C. albicans* in a mouse model, although the deletion of *SOD5* gene showed no effect in *C. albicans* killing by macrophages. It has also been implicated in the degradation of reactive oxygen species generated by the host to evade surveillance of the innate immune system, resistance to miconazole through biofilm formation, and defense against host insults. Other protein that was also extracted with SDS and zymolyase was the extracellular glycosidase CRH11, a glycosylphosphatidylinositol-anchored protein involved in cell wall organization [75]. Tsa1-B was exclusively found in the fraction of proteins extracted with chitinase treatment. Tsa1-B is a peroxiredoxin, a thiol-specific peroxidase that plays a protective role against reactive oxygen species, organization of the cell wall [76], and is essential for the yeast-to-mycelium transition [77].

The analysis of all SDS-extracted proteins [19] in the PANTHER classification system according the cellular component revealed that 518 proteins (43.2%) correspond to cell part, two proteins (0.2%) belong to the extracellular region, 254 proteins (21.2%) are part of macromolecular complexes, 119 (9.9%) are membrane proteins, one (0.1%) belongs to the nucleoid, and

Nuclear proteins	U6 snRNA-associated SM-like protein LSM1 and LSM4; RuvB-like helicases 1 (RVB1) and 2 (RVB2); replication factor C subunit 3 RFC3; nuclear pore complex protein NUP50; histones H2AZ, H2A.1, H2A.2, H3.3, and H4; histone deacetylase complex subunit SAP30L; high mobility group protein DSP1; transcription elongation factors SPT5 and SPT6; mediator of RNA polymerase II transcription subunits 10 (NUT2) and 4 (MED4); DNA-directed RNA polymerase II subunits RPB2, RPB7 and RPB8; single-stranded telomeric DNA-binding/mRNA-binding protein GBP2; DNA-directed RNA polymerase I subunit RPA49; negative cofactor 2 transcription regulator complex subunit HFL2; DNA-directed RNA polymerase III core subunit RPC19 and RPC40; RNA polymerase-associated protein LEO1 homolog; transcription factor IWS1; snoRNP complex protein SIK1; nucleolar protein 58 NOP58; karyopherin β (importin subunit β) and karyopherin β-3 (importin subunit β); importin subunit α; restriction of telomere capping protein 3; U3 small nucleolar RNA-associated protein 22 UTP22; decapping protein 1, isoform a	U6 snRNA-associated SM-like protein LSM1 and LSM4; RuvB-like helicases 1 (RVB1) and 2 (RVB2); replication factor C subunit 3 RFC3; nuclear pore complex protein NUP50; histones H2AZ, H2A.1, H2A.2, H3.3 and H4; histone deacetylase complex subunit SAP30L; high mobility group protein DSP1; transcription elongation factors SPT5 and SPT6; mediator of RNA polymerase II transcription subunits 10 (NUT2) and 4 (MED4); DNA-directed RNA polymerase II subunits RPB2, RPB7 and RPB8; single-stranded telomeric DNA-binding/mRNA-binding protein GBP2; DNA-directed RNA polymerase I subunit RPA49; negative cofactor 2 transcription regulator complex subunit HFL2; DNA-directed RNA polymerase III core subunit RPC19 and RPC40; RNA polymerase-associated protein LEO1 homolog; transcription factor IWS1; snoRNP complex protein SIK1; nucleolar protein 58 NOP58; Karyopherin β (Importin subunit β) and Karyopherin β-3 (Importin subunit β); Importin subunit α; restriction of telomere capping protein 3; U3 small nucleolar RNA-associated protein 22 UTP22; decapping protein 1, isoform a

Proteasome	Proteasome endopeptidase complex subunits PRE8 (proteasome subunit α type 2) and PRE9 (proteasome subunit α type 4); proteasome subunit β, proteasome regulatory particle base subunit RPN2 (26S proteasome non-ATPase regulatory subunit 1); proteasome regulatory particle lid subunit (26S proteasome non-ATPase regulatory subunit 8); proteasome regulatory particle base subunit RPN10 (26S proteasome non-ATPase regulatory subunit 4); proteasome regulatory particle lid subunit RPN5 (26S proteasome non-ATPase regulatory subunit 12); proteasome core particle subunit beta 1 PRE3; proteasome endopeptidase complex PRE10 (proteasome subunit α type 3); proteasome endopeptidase complex PRE5 (proteasome subunit α type-related); proteasome assembly chaperone 2; proteasome core particle subunit β4; proteasome core particle subunit β6; proteasome regulatory particle base subunit RPN1; proteasome endopeptidase complex PRE6; proteasome endopeptidase complex PUP2; 26S proteasome non-ATPase regulatory subunit 13 (proteasome regulatory particle lid subunit); proteasome endopeptidase complex SCL1 (proteasome subunit α type-6).	Proteasome endopeptidase complex subunits PRE8 (proteasome subunit α type 2) and PRE9 (proteasome subunit α type 4); proteasome subunit β, proteasome regulatory particle base subunit RPN2 (26S proteasome non-ATPase regulatory subunit 1); proteasome regulatory particle lid subunit (26S proteasome non-ATPase regulatory subunit 8); proteasome regulatory particle base subunit RPN10 (26S proteasome non-ATPase regulatory subunit 4); proteasome regulatory particle lid subunit RPN5 (26S proteasome non-ATPase regulatory subunit 12); proteasome core particle subunit beta 1 PRE3; proteasome endopeptidase complex PRE10 (proteasome subunit α type 3); proteasome endopeptidase complex PRE5 (proteasome subunit α type-related); proteasome assembly chaperone 2; proteasome core particle subunit β4; proteasome core particle subunit β6; proteasome regulatory particle base subunit RPN1; proteasome endopeptidase complex PRE6; proteasome endopeptidase complex PUP2; 26S proteasome non-ATPase regulatory subunit 13 (proteasome regulatory particle lid subunit); proteasome endopeptidase complex SCL1 (proteasome subunit α type-6)
Endoplasmic Reticulum	α-1,2-Mannosidase (mannosyl-oligosaccharide 1,2-α-mannosidase) MNS1; endoplasmic reticulum vesicle protein 25 (ERV5); UDP-glucose:glycoprotein glucosyltransferase Kre5; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 OST1; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit WBP1; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2; dolichyl-phosphate-mannose-protein mannosyltransferase 2 PMT2; signal peptidase complex subunit SPC2; signal peptidase complex catalytic subunit Sec11.	α-1,2-Mannosidase (mannosyl-oligosaccharide 1,2-α-mannosidase) MNS1; endoplasmic reticulum vesicle protein 25 (ERV5); UDP-Gucose:glycoprotein glucosyltransferase Kre5; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 OST1; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit WBP1; dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2; dolichyl-phosphate-mannose-protein mannosyltransferase 2 PMT2; signal peptidase complex subunit SPC2; signal peptidase complex catalytic subunit Sec11
Golgi Apparatus	SNAP receptor complex member 1; Golgi phosphoprotein 3 Sauron homolog; α-1,6-mannosyltransferase HOC1 α-1,2-mannosyltransferase MNT1	SNAP receptor complex member 1; Golgi phosphoprotein 3 Sauron homolog; α-1,6-mannosyltransferase HOC1 α-1,2-mannosyltransferase MNT1
Secretion	Protein transport proteins Sec61 subunit β, Sec31; translocation protein Sec63; coatomer subunit γ2 Sec21; Ras-related protein Sec4; signal peptidase complex catalytic subunit Sec11	Protein transport proteins Sec61 subunit β, Sec31; translocation protein Sec63; coatomer subunit γ2 Sec21; Ras-related protein Sec4; signal peptidase complex catalytic subunit Sec11
Vacuole	V-type proton ATPase subunit a VPH1; V-type proton ATPase catalytic subunit A TFP1; V-type proton ATPase subunit C VMA5; H(+)-transporting V0 sector ATPase subunit d	V-type proton ATPase subunit a VPH1; V-type proton ATPase catalytic subunit A TFP1; V-type proton ATPase subunit C VMA5; H(+)-transporting V0 sector ATPase subunit d

Cytoskeleton-related proteins	CDC10 cell-division-related protein; actin-related protein 2/3 complex subunit 3 (ARC18), subunit 4 (ARC19) and subunit 5 (ARC15); MLC1 (a protein related to cytoskeleton with function in the cellular bud neck contractile ring); septin CDC3 and septin CDC12; dynamin-like GTPase VSP1 (vacuolar protein sorting-associated protein 1); SLP2 (stomatin-like protein 2 mitochondrial); tubulin α chain TUB1; tubulin β chain TUB2; fimbrin SAC6; actin ACT1	CDC10 cell division related protein; Actin-related protein 2/3 complex subunit 3 (ARC18), subunit 4 (ARC19) and subunit 5 (ARC15); MLC1 (a protein related to cytoskeleton with function in the cellular bud neck contractile ring); Septin CDC3 and Septin CDC12; Dynamin-like GTPase VSP1 (vacuolar protein sorting-associated protein 1); SLP2 (Stomatin-like protein 2 mitochondrial); Tubulin α chain TUB1; Tubulin β chain TUB2; Fimbrin SAC6; Actin ACT1
Ribosomal and mitochondrial proteins	37S ribosomal proteins S9 and MRP51; 39S ribosomal protein L21; NAD-dependent malic enzyme; Protein FYV4 mitochondrial; 28S ribosomal proteins S7, S18C, S23, S29 and S36; aconitate hydratase; Mitochondrial 54S ribosomal protein YmL7/YmL5; 54S ribosomal protein L3; 39S ribosomal proteins L13, L15, L19, L24, L27, L40, L41, L43, and L49; aspartate transaminase; aspartate aminotransferase AAT22; cytochrome c peroxidase; D-lactate dehydrogenase; enoyl-(acyl-carrier-protein) reductase ETR mitochondrial; enoyl-(acyl-carrier-protein) reductase MRF1 mitochondrial; TOM20; COX assembly protein homolog (UniProtKB ID_Q5ALU3); 37S ribosomal proteins S8, S10, S16, and S17; 39S ribosomal proteins L11, L27, and L43; ATP synthase assembly factor FMC1; succinate dehydrogenase assembly factor 4; HSP60 mitochondrial; arginine-tRNA ligase; succinate dehydrogenase assembly factor 2; HSP10 mitochondrial; aconitate hydratase; 4-aminobutyrate transaminase UGA1; acetolactate synthase regulatory subunit ILV6; TOM40; mitochondrial outer membrane protein OM14; 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase mitochondrial COQ5; H/ACA ribonucleoprotein complex subunit 1 GAR1; cytochrome c CYC1; α-ketoglutarate dehydrogenase KGD1; mitochondrial fission 1 protein FIS1; altered inheritance of mitochondria protein 19.	37S ribosomal proteins S9 and MRP51; 39S ribosomal protein L21; NAD-dependent malic enzyme; protein FYV4 mitochondrial; 28S ribosomal proteins S7, S18C, S23, S29 and S36; aconitate hydratase; mitochondrial 54S ribosomal protein YmL7/YmL5; 54S ribosomal protein L3; 39S ribosomal proteins L13, L15, L19, L24, L27, L40, L41, L43, and L49; aspartate transaminase; aspartate aminotransferase AAT22; Cytochrome c peroxidase; D-lactate dehydrogenase; enoyl-(acyl-carrier-protein) reductase ETR mitochondrial; enoyl-(acyl-carrier-protein) reductase MRF1 mitochondrial; TOM20; COX assembly protein homolog (UniProtKB ID_Q5ALU3); 37S ribosomal proteins S8, S10, S16 and S17; 39S ribosomal proteins L11, L27 and L43; ATP synthase assembly factor FMC1; Succinate dehydrogenase assembly factor 4; HSP60 mitochondrial; Arginine-tRNA ligase; Succinate dehydrogenase assembly factor 2; HSP10 mitochondrial; aconitate hydratase; 4-aminobutyrate transaminase UGA1; acetolactate synthase regulatory subunit ILV6; TOM40; mitochondrial outer membrane protein OM14; 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase mitochondrial COQ5; H/ACA ribonucleoprotein complex subunit 1 GAR1; Cytochrome c CYC1; α-ketoglutarate dehydrogenase KGD1; mitochondrial fission 1 protein FIS1; altered inheritance of mitochondria protein 19

Table 1. Proteins found in several intracellular compartments that were identified in the 2% SDS-extracted cell wall fraction by tandem mass spectrometry according to [29].

304 (25.4%) to organelles. Proteins related to nucleus, proteasome, endoplasmic reticulum, Golgi apparatus, secretion, vacuole, cytoskeleton, and mitochondria are shown in **Table 1**.

The zymolyase-extracted proteins were classified in cellular component as follows: 18 proteins (48.6%) to cell part, one (2.7%) to extracellular region, nine (24.3%) to macromolecular complex, one (2.7%) to membrane, and eight (21.6%) to organelles. The proteins solubilized

with chitinase were classified in cell part (23, 43.4%), macromolecular complex (14, 26.4%), membrane proteins (3, 5.7%), and from organelles (13, 24.5%). Three proteins were exclusively found in the fraction of proteins extracted with zymolyase, including the cell surface Cu-only superoxide dismutase 5 (SOD5), the extracellular glycosidase CRH11, and the covalently linked cell wall protein 14 (SSR1 or CCW14).

According to our results and those from other groups, the cell wall of *C. albicans* contains many intracellular proteins that are commonly found in nucleus, cytoplasm, endoplasmic reticulum, Golgi apparatus, and vacuole. Many of these proteins have no signal peptide for entry into the classic secretory pathway involving the endoplasmic reticulum and the Golgi apparatus. For these type of proteins, an unconventional secretory pathway has been reported [19, 55, 78–81]. Some of these proteins are glycolytic enzymes, which perform different functions inside and outside the cell [82], including enolase and phosphoglucose isomerase, which do not have enzymatic activity outside the cell.

The secretory pathway of glycolytic enzymes is yet to be characterized. This pathway seems to be nonconventional since glycolytic enzymes have no secretion signal. One of this nonconventional pathways has been revealed for the acyl coenzyme A-binding protein (AcbA) of *Dictyostelium discoideum*, which has full requirement of the Golgi reassembly stacking protein (GRASP), a protein attached to the cytosolic face of the Golgi apparatus membrane [83]. The Acb1 protein of *S. cerevisiae* also requires the yeast GRASP protein for its secretion, which is also mediated by proteins involved in autophagy and Sso1 protein, a t-SNARE localized in the plasma membrane [84]. The *S. cerevisiae* Eno2 protein does not possess a secretion signal sequence, since Eno2 and Pgi1 proteins were secreted in a *sec23–1* mutant at 37°C. These data provide evidence that the secretion of some glycolytic enzymes is independent of the conventional secretory pathway. Analysis of deletion mutants showed that the knock-in of the SNARE coding *TLG2* gene inhibits the enolase secretion. However, other proteins might be involved, since the inhibition of secretion is not complete [85]. One of the best studied nonconventional secretion pathways is the secretion of the a-factor mating pheromone, which has a full requirement of the Ste6 protein, a transport protein with homology to MDR proteins [86]. Other protein that does not follow the classical secretion pathway is galectin-1, a mammalian lactose-binding lectin that was expressed in yeast [87]. The galectin-1 expression was dependent on the yeast genes *NCE101*, *NCE102*, *NCE103*, while the corresponding *C. albicans* are XP_019330961.1(CaNCE1), XP_723171.1 (CaNCE2), and XP_721792.1(CaNCE3, also named carbonate dehydratase), and therefore, the existence of this protein export pathway is possible in *C. albicans.*

Results obtained by mass spectrometry of the cell walls of *C. albicans*, *C. glabrata*, *C. krusei,* and *C. parapsilosis* exposed to different concentrations of H_2O_2 have allowed the identification of wall proteins known as multifunctional or *moonlighting*. This class of proteins includes: (i) glycolytic enzymes, (ii) thermal shock, (iii) oxidative stress response proteins, (iv) general metabolic enzymes, and highly conserved proteins, being over- or subexpressed in the presence or absence of reactive species of oxygen (ROS). Some of the identified glycolytic enzymes are the triphosphate isomerase (Tpi1), glyceraldehyde 3-phosphate dehydrogenase (GADPH), fructose bisphosphate aldolase (Fba1), phosphoglycerate kinase (Pgk), phosphoglycerate mutase (Gpm1), pyruvate kinase (Pk), and enolase 1 (Eno1) [88].

Finally, we want to call the attention to the fact that ^{14}C-labeled proteins labeled by TGase activity and extracted with SDS showed a molecular mass lower than 50 kDa [19]. However, the theoretical molecular mass of many of them was in the range between 50 and 220 kDa. Therefore, fragments of these proteins are being transported to the extracellular milieu and can be cross linked each other, either to β-1,3- or β-1,6-glucan, or chitin to strengthen the protection properties of the cell wall. This observation was evidenced by the inhibition of TGase by cystamine that facilitated the action of zymolyase on *C. albicans* yeasts with the consequent reduction of the osmoprotective properties of the cell wall [19]. However, more studies are needed to support this hypothesis.

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Section 3

Interaction of Cells Host and Other Microbe

Chapter 5

The Role of Phagocytes in Immunity to *Candida albicans*

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Additional information is available at the end of the chapter

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Abstract

Body clearance of fungi such as *Candida albicans* involves phagocytosis by fixed tissue macrophages as well as infiltrating monocytes and neutrophils. Through phagocytosis, the fungi are confined and killed by the oxidative and non-oxidative anti-microbial systems. These include oxygen derived reactive species, generated from the activation of the NADPH oxidase complex and granule constituents. These same mechanisms are responsible for the damage to hyphal forms of *C. albicans*. Complement promotes phagocytosis, through their interaction with a series of complement receptors including the recently described complement receptor immunoglobulin. However, it is also evident that under other conditions, the killing of yeast and hyphal forms can occur in a complement-independent manner. Phagocytosis and killing of *Candida* is enhanced by the cytokine network, such as tumour necrosis factor and interferon gamma. Patients with primary immunodeficiency diseases who have phagocytic deficiencies, such as those with defects in the NADPH oxidase complex are predisposed to fungal infections, providing evidence for the critical role of phagocytes in anti-fungal immunity. Secondary immunodeficiencies can arise as a result of treatment with anti-cancer or other immunosuppressive drugs. These agents may also predispose patients to fungal infections due to their ability to compromise the anti-microbial activity of phagocytes.

Keywords: *Candida albicans*, macrophages, neutrophils, complement, innate immunity, phagocytosis, fungal killing mechanisms, cytokines, trained immunity, immunodeficiency, immunopharmacology

1. Introduction

C. albicans is considered to be the most common fungus causing both skin and disseminated disease, particularly in immunodeficient and immunocompromised patients. Phagocytes, particularly neutrophils, play an important role in clearing candidal infections. The importance of neutrophils in immunity to *C. albicans* is clearly evident from the increased rate of infection seen in patients with severe neutropenia [1].

In neutrophils, the major response associated with phagocytosis of microbial pathogens is the oxygen-dependent respiratory burst and the generation of reactive oxygen species (ROS). Several decades ago it became evident that neutrophils displayed a unique respiratory burst in the absence of mitochondria, where the generation of ATP comes mainly from glycolysis (reviewed in [2]). It also became apparent that the majority of the oxygen consumed was converted to superoxide ($O\cdot_2^-$) which is then converted to further oxygen intermediates, including singlet oxygen and H_2O_2. The enzyme which catalyses the conversion of O_2 to $O\cdot_2^-$ is assembled in the phagocytic vacuole membrane, facilitating its release into the bacteria or fungus-containing vacuole. In neutrophils, the release of the azurophilic granule content simultaneously into the phagocytic vacuole leads to the generation of HOCl, a highly potent anti-microbial agent, as a result of the action of myeloperoxidase on H_2O_2 in the presence of chloride ions. In addition, ingestion of microbial pathogens and their confinement to the vacuolar environment may restrict the supply of essential nutrients necessary for growth.

The NADPH oxidase complex is responsible for the respiratory burst and consists of a number of different proteins which assemble in the neutrophil vacuole membrane following cell stimulation. This is typically initiated during phagocytosis of bacteria and fungi [3]. The complex consists of the oxidase-specific phox proteins $gp91^{phox}$, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the small GTPases, Rac1 and Rac2. Cell activation leads to the assembly of these components in the membrane and the initiation of enzymatic activity.

The non-oxidative microbicidal system complements the respiratory burst. Components of the azurophilic granules in neutrophils have been shown to have anti-microbial activity. These include defensins, serprocidins and bactericidal/permeability increasing protein (BPI). Defensins are cationic peptides with broad spectrum antimicrobial activity [2]. The seroprodins, elastase, azurocidin and cathepsin G have antimicrobial activity independent of their enzymatic activity [2].

As with neutrophils, most bacteria and fungi are confined and killed within phagosomes by macrophages [4], involving a variety of agents such as toxic metabolites, peptides and enzymes. These may act either alone or synergistically. In addition, macrophages can produce ROS which have anti-microbial action but unlike monocytes, macrophages lack MPO. Most striking is the marked heterogeneity of macrophages enabling these leukocytes to perform functions relevant to specific tissues in which they are located.

The extrusion of neutrophil extracellular traps (NETs) is also considered to be a defence mechanism against microbial pathogens. NETs are structures composed of DNA as well as anti-microbial substances, elastase, calprotectin and MPO [5]. NETs not only trap the microbial pathogens, but also kill them. Interestingly, it has been reported that the formation of NETs requires the presence of ROS [6].

Effective recognition of microbial pathogens by neutrophils and macrophages requires receptors which bind peptides deposited on bacterial and fungal surfaces which have been generated through the activation of complement, namely C3b and iC3b. Receptors recognising iC3b include CR3 (CD18/CD11b) and CR4 (CD18/CD11c), which are present on both neutrophils and macrophages. Recently, another complement receptor type, complement immunoglobulin receptor (CRIg), expressed only by a subpopulation of macrophages has been described, which binds both iC3b and C3b (reviewed in [7]). It has been shown that this receptor plays an important role in clearance of bacteria from the circulation by liver Kupffer cells [8] and may also be a pattern recognition receptor, facilitating clearance of bacteria in the absence of complement [9].

Antibody bound to microbial pathogens also promotes phagocytosis through the Fcγ receptors, FcγRI (CD64), FcγRIIA (CD32) and FcγRIIIB (CD16), all of which engage the Fc domain of Immunoglobulin G (IgG). The FcαRI which binds the Fc domain of Immunoglobulin A (IgA) also promotes microbial phagocytosis and killing [2].

Apart from the integrins and FcγRs, neutrophils and macrophages express a range of pattern recognition receptors (PPR) which recognise conserved microbial pathogen structures, such as lipoteichoic acid, β-glucans and lipopolysaccharide. Families of PPRs include those found in serum (pentraxins, collectins, complement), those which are membrane bound (classic C-type lectins, non-classic C-type lectin leucine-rich proteins, scavenger receptors) and those which are located intracellularly (NODs, interferon induced proteins).

2. Complement dependent and independent phagocytosis of *C. albicans*

Despite the importance of complement-independent mechanisms for host anti-candidal immunity, it is evident that complement is required for optimal resistance to fungal infection [10–12]. It was also evident in these studies that complement could be activated by *C. albicans* by the alternative pathway. Activation of complement leads to the generation of chemotactic peptides and C5a, which attracts neutrophils to the site of candidal infection [13, 14]. Thong and Ferrante [11], in their studies on the generation of chemotaxis promoting factors by serum treated with *C. albicans*, showed that this activity was totally dependent on heat-labile factors and activation of complement via the alternative pathway. Chemotaxis of neutrophils towards fungus-treated serum was abolished when the serum was either heated at 56°C for 20 min or was C2 deficient (where the alternative but not the classical pathway can be activated). The subsequent step, phagocytosis, was also highly dependent on heat labile opsonins [12]. However, while the chemotactic response was totally dependent on serum complement, the heat labile opsonins only acted to enhance other phagocytosis-promoting mechanisms. Thus, significant phagocytosis was still observed in the presence of heat-inactivated serum. In both of these studies on chemotaxis and phagocytosis-promoting activity of serum, it was shown that these principles applied to a wide-range of clinical isolates of *C. albicans* from patients and both including Serotypes A and B [11, 12].

Zymosan A is a yeast cell wall glucan and, like *C. albicans* derived β(1,3) (1,6)-glucan, is an agonist to TLR2 and Dectin-1 [15]. Using commercially available labelled zymosan A bioparticles

which are non-fluorescent outside of the cell and fluoresce once taken into acidic phagosomes, we showed that neutrophils require opsonising conditions to phagocytose particles efficiently (**Figure 1**). This supports the findings of [16], and demonstrates that like monocytes and macrophages, neutrophils require complement for the rapid phagocytosis of yeast particles. Interestingly, the complement dependency of phagocytosis diminished at incubation times of 45–60 min, where complement-independent mechanisms of phagocytosis become more prominent (**Figure 1C**).

The classical complement pathway is likely to be activated by mannan-specific antibodies found in human serum [19] whereas the lectin pathway is activated by the binding of mannose-binding lectin to mannan on the cell wall of the fungus [20]. However, it has also been shown that *C. albicans* can bind the complement regulatory protein, C4b-binding protein (C4BP), thereby inactivating C4b and hence preventing complement activation on the yeast surface. As a result, the microbial pathogen will evade complement activation via the classical and lectin pathways, but the alternative pathway remains operative, generating chemotactic factors and opsonins. Furthermore, *C. albicans* has the ability to regulate the alternative pathway and factors H and FHL-1 [21]. The binding of these regulators is seen with both the cellular and hyphal forms of *C. albicans* [22].

The unique complement receptor CRIg is a member of the transmembrane protein of the type 1 immunoglobulin superfamily, encoded by *VSIG4*. Although two spliced forms of CRIg have been described, a long (L) and short (S) form [8], we have recently identified five forms based on expression of transcripts and western blot analysis [23]. The receptor is expressed selectively by a subpopulation of macrophages, probably of the M2 type, and is abundant in fixed tissue macrophages such as liver Kupffer cells and resident peritoneal macrophages [24, 25]. Unlike CR3 and CR4 which require prior activation, CRIg is naturally active and its activity is controlled by its recycling pattern from the endoplasmic reticulum [8]. Our studies have demonstrated that cytokines alter CRIg expression in human macrophages and this was associated with a corresponding change in ability of neutrophils to phagocytose *C. albicans* in a complement-dependent manner [23, 26, 27].

While CR3 and FcRγ mediate phagocytosis of complement and antibody opsonised *C. albicans*, in the absence of these opsonins, adherence and phagocytosis by neutrophils and macrophages is promoted by C-Type Lectin Receptors (CLRs), in particular Dectin-1 [28–31]. The targets for Dectin-1 are β-1,3 glucan polymers, major components of the fungal cell wall. In *C. albicans* hyphae, this polymer is masked and appears to be different in the yeast form [32].

Cells of the phagocytic system are able to recognise *C. albicans* through multiple classes of receptors [33]. These include pattern recognition receptors (PRRs) such as Toll-like receptor (TLRs) 4 and 2 [34, 35], and CLRs such as Dectin-1 and the mannose receptor [36]. While these receptors are able to induce phagocytosis independently of complement, efficiency of uptake in both macrophages and neutrophils can be significantly increased when *C. albicans* is opsonised [16]. Under these conditions CR3 present on phagocytes is able to recognise iC3b deposited on the fungal cell surface and promote phagocytosis. In macrophages, this process is also able to occur through CRIg [27]. Agents such as dexamethasone that promote the upregulation of CRIg protein expression are also able to induce increased levels of phagocytosis of *C albicans* [23], suggesting that CRIg rather than CR3 plays an important role in the phagocytosis of *C. albicans* in macrophages.

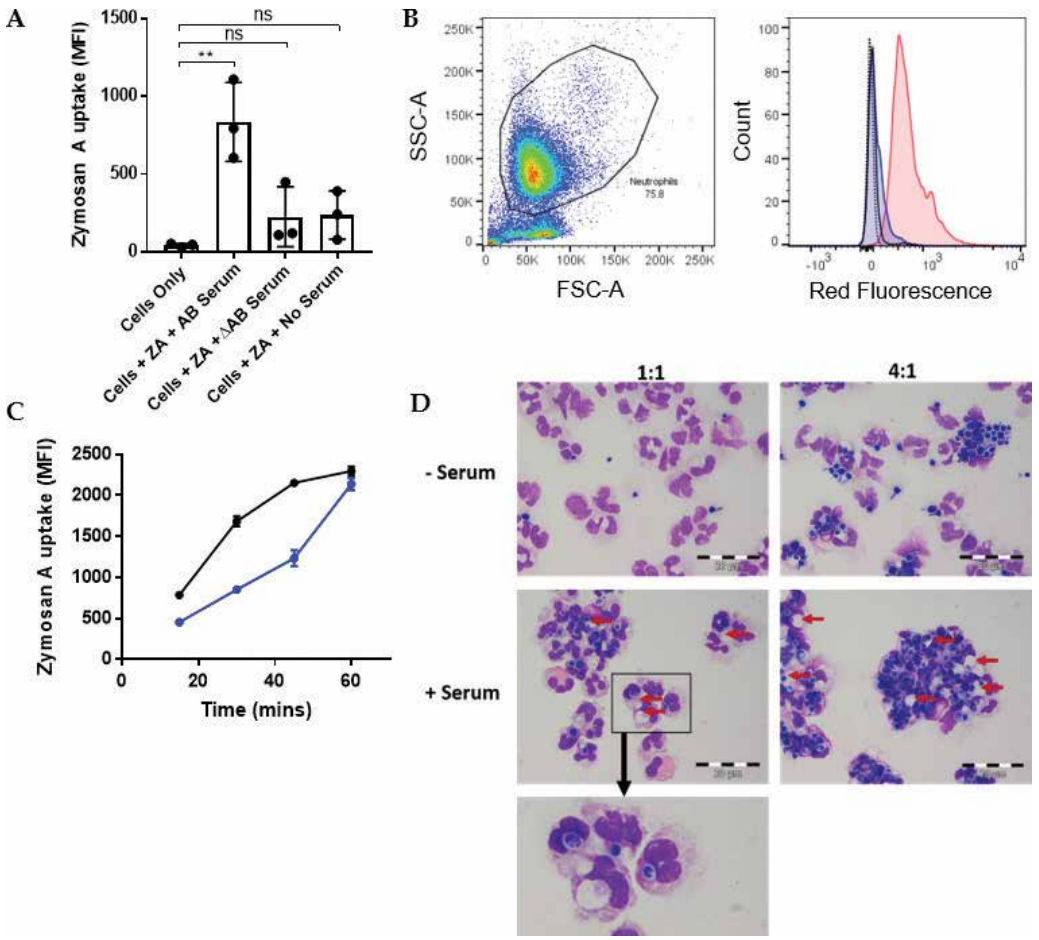

Figure 1. Complement-dependent and -independent phagocytosis of zymosan A bioparticles by human neutrophils. (A) Phagocytosis of *C. albicans* under the different treatment conditions indicated in the x-axis. Results are the mean ± SD of three experiments. (B) Representative histogram/gating strategy for these experimental runs. In these experiments the reaction was terminated at 30 min. Neutrophils only are shown by the dashed line, no serum in black, heat-inactivated serum shown in blue, and native AB serum shown in red. (C) Phagocytosis kinetics over a 60 min incubation period in the presence or absence of serum. Results are presented as mean ± SD of triplicate reactions. Neutrophils were prepared from human peripheral blood from healthy volunteers, using the high density gradient method [17]. Phagocytosis was assayed using pHrodo™Red Zymosan A bioparticles (ThermoFisher, Walter MA, Cat no. P35364) as described previously [18]. Human AB serum was prepared from peripheral blood of healthy volunteers. The serum was shown to have normal levels of complement activity using the CH50 assay. AB serum heated at 56°C for 20 min was confirmed to lack complement activity. The cell samples were analysed using a FACSCanto I flow cytometer (BD). The work was approved by the Human Research Ethics Committee of the Women's and Children's Hospital Network, Adelaide. Statistical analyses were carried out by ANOVA followed by Dunnet's post hoc test. (D) Photomicrographs of the interaction of *C. albicans* with neutrophils in the presence or absence of serum at a 1:1 and 1:4 neutrophil:fungal ratio. Red arrows indicate phagocytic vacuoles following the digestion of the yeast or non-degraded yeast particles (following 30 min of incubation).

Neutrophils recognise *C. albicans* through the PRRs TLR2, TLR4 and Dectin-1, and also under opsonising conditions through FcγR and CR3 [37]. Similar to macrophage phagocytosis of *C. albicans*, uptake of isolated fungal zymosan A is more efficient in opsonising conditions, with phagocytosis after a 15-min incubation time being three times higher in reactions with complement compared to no serum and heat-inactivated serum controls (**Figure 1**).

C. albicans is able to exist in multiple forms, as a single-celled budding yeast or in pseudohyphal or hyphal filamentous forms [38]. While in its unicellular form, the fungus can be tolerated as a commensal organism by the oral or vaginal epithelium. However, when it converts to its hyphal form, the fungus displays pathogenic properties. The host is able to discriminate against the potential danger [37] through MAPK-based recognition in the epithelial cells [39], which leads to mitogen-activated protein kinase phosphatase 1 (MKP1) and c-Fos activation. Neutrophils also play a role in this protection through TLR4-mediated recognition [40].

3. Trained macrophage immunity in anti-fungal immunity

Trained immunity (TI) refers to the ability of innate immune cells to exhibit 'memory' and prevent reinfection of previously encountered invading pathogens [41]. Termed by Netea and colleagues [42], TI induces a state of enhanced antimicrobial action in cells of the innate immune system, particularly monocytes and macrophages, which is distinct from both typical innate immunity and the memory of the adaptive immune system. Alternatively, TI refers to the enhanced response to reinfection against the initial invading microorganisms and cross-protection against different pathogens. Although the concept of TI is relatively new, the phenomenon of protection afforded by previous infection in a manner distinct from adaptive immunity has long been known, particularly in plant and insect systems [43, 44].

TI has been shown to have a role in infection and immunity against *C albicans.* Bistoni et al. [45] demonstrated that not only did injection with a non-pathogenic strain of *C. albicans* induce protection against reinfection, but also cross-protected against the other pathogens *Candida tropicalis* and *Staphylococcus aureus*. This protection was determined to be macrophage-dependent, as transfer of adherent splenic cells from mice administered with the non-pathogenic strain conferred protection to the recipient mice. Two decades later, Quintin et al. [46] expanded on this concept, demonstrating that mice injected with low doses of *C. albicans* showed increased survival rates when administered lethal infection loads, and increased proinflammatory cytokine production upon secondary exposure. This protection was also shown in mice deficient in T and B cells and not in mice lacking *CCR2*, indicating that similar to the results of Bistoni et al. [45], the observed protection was monocyte-dependent. It was also shown that training of monocytes could be induced through purified β-glucan, a polysaccharide that makes up the cell wall of selected bacteria and fungi [47]. The group further investigated the mechanisms behind this protection by analysis of the genome-wide binding pattern of the methylation marks on histone 3 lysine 4 (H3K4me3) and on histone 3 lysine 27 (H3K27me3), and concluded that protection was controlled at the epigenetic level through H3K4me3 in known genes involved in innate immunity. Furthermore, mRNA levels of TNF and IL-6 were higher in trained monocytes compared with non-trained control cells.

While other molecules such as fungal chitin have also been shown to induce TI [48], β-glucan remains the most well-studied molecule with respect to *C. albicans*, which has been shown to induce TI in both human and murine systems [46, 49, 50]. Along with its antimicrobial priming, β-glucan-induced TI has also been investigated in anti-tumour immunity [51].

4. Killing of *C. albicans* by neutrophils and macrophages

Ferrante [52] demonstrated that killing of yeast forms of *C. albicans* and *Candida glabrata* was associated with release of the ROS, superoxide, and constituents of azurophilic granules and specific granules. During this interaction the generation of HOCl occurred, another potent anti-fungal agent. The importance of ROS was demonstrated by the finding that inhibitors of superoxide and H_2O_2 decreased intracellular killing of *C. albicans* [53]. Further proof of the role of ROS generation in the killing of *C. albicans* came from the demonstration that neutrophils and macrophages from patients with chronic granulomatous disease (CGD) (who have defective NADPH oxidase activity), were unable to effectively kill the fungi [54]. However, whether ROS *per se* are responsible for the killing of *C. albicans* remains to be established [55]. The reaction of H_2O_2 with MPO, in the presence of chloride ions, forms a very potent antimicrobial system. We have previously demonstrated that opsonised *C. albicans* induces the release of both H_2O_2 and MPO, thereby establishing an anti-candidal environment [52]. The importance of MPO in the killing of *C. albicans* is supported by the finding that neutrophils and monocytes from MPO deficient patients failed to kill *C. albicans* [56, 57].

In vivo the absence of MPO in macrophages may be overcome by the cells incorporating MPO released by neutrophils at infection sites. Thus, resident peritoneal mouse macrophages in the presence of recombinant MPO caused an increase in intracellular killing of *C. albicans* [58]. However, it is noteworthy that using mouse models of X-linked CGD and MPO deficiency, susceptibility was most evident in the former, suggesting that ROS are the major mediators of candidicidal activity [59]. In comparison, the neutrophil-mediated damage to *C. albicans* pseudohyphae was found to be mediated by the oxidative burst and MPO [60]. Interestingly, this neutrophil-mediated damage occurred in the absence of serum complement.

Two distinct mechanisms for human neutrophil-mediated killing have been documented, depending on the state of fungal opsonisation. Using *in vitro* fungicidal assays, Gazendam et al. [61] showed that killing of un-opsonised *C. albicans* was dependent on CR3 and phosphatidylinositol-3-kinase (PI3K) signalling, but was independent of NADPH oxidase activation. However, the killing of antibody opsonised *C. albicans* by neutrophils was dependent on Fcγ receptors and protein kinase C (PKC) in addition to NADPH.

5. Intracellular signalling required for killing of *C. albicans*

Approximately two decades ago it was demonstrated that human neutrophil-mediated killing of *C. albicans*, in a complement-dependent manner, required the activation of the extracellular signal-regulated protein kinase cascade [62]. More recently it has been reported that PKCδ activation downstream of the receptors Dectin-1 and Mac-1 is important in the neutrophil-mediated resistance to *C. albicans* and fungi-induced ROS generation [63]. In contrast, while PKCδ deficiency in macrophages prevented the stimulation of production of ROS induced by *C. albicans*, this did not affect the killing of the fungus. It has been demonstrated that BTK and

Vav1 are Dectin-1 interacting proteins [64]. These were found to be recruited to phagocytic cups containing yeast or hyphae of *C. albicans*, at the less mature stage of phagosome development. These contribute to the Dectin-1 dependent phagocytosis of *C. albicans*.

In comparison, Gazendam et al. [61] demonstrated that neutrophils display two different mechanisms in the killing of *C. albicans* by evaluating patients with Dectin-1 deficiency, CARD9 deficiency or NADPH deficiency. One of these mechanisms was CR3, PI3K and CARD9 dependent, but independent of ROS generation. The other was selectively dependent on Fcγ, PKC and ROS generation. Both of these candidicidal pathways required Syk tyrosine activation but were independent of Dectin-1.

6. Neutrophil extracellular traps in immunity to *C. albicans*

C. albicans has been shown to induce NET extrusion in phagocytes, particularly in neutrophils. While the formation of this structure is considered as part of cell death or NETosis [65], Byrd et al. [66] reported that the rapid extrusion of NETs in response to *C. albicans* occurs in the absence of cell death. However, others have demonstrated that the yeast forms of *C. albicans* stimulated NETs through autophagy and ROS generation in the early stage of the interaction (first 15 min) [67]. However, with the hyphal forms, NET formation occurred via autophagy and not ROS generation. In the longer term (4 h), only the hyphae stimulated NETs. Interestingly, they found less killing of yeast forms by NETs compared to the high level of damage to the hyphae forms. Other strategic functions of extracellular protrusions of neutrophils have been demonstrated for *Plasmodium falciparum*. Here, the neutrophils were observed to 'throw out' protrusions which penetrated the parasitophorous vacuole containing the intraerythrocytic stage of the parasite and withdrawing the parasite without damaging the erythrocyte [68].

7. Cytokine priming in phagocyte-mediated killing of *C. albicans*

Over three decades ago it became evident that neutrophil responses to microbial pathogens could be significantly increased if the cells were pre-sensitised with products released by activated lymphocytes and macrophages [69], a process dependent on the presence of TNF [70, 71]. The importance of cytokine priming in killing of *C. albicans* by neutrophils was also observed [72]. Thus, neutrophil mediated killing of *C. albicans* and a related fungus, *Candida glabrata* was significantly increased if the phagocytes had been pre-treated with either TNF or GM-CSF [52, 73]. The TNF treatment also increased the candida-induced release of ROS and MPO, consistent with the increased anti-fungal activity induced by the cytokines [52]. The mechanism by which TNF primes neutrophils for increased killing of *C. albicans* has not been studied. However, these mechanisms can be inferred from studies with other microbial pathogens. Kowanko et al. [74] demonstrated that the TNF-induced effects responsible for increased microbial killing could be mediated by both oxygen-dependent and oxygen-independent mechanisms, with respect to killing of opsonised *S. aureus* and *Plasmodium falciparum* infected erythrocytes, respectively. Furthermore, studies with the pathogenic soil amoeba,

N. fowleri have shown that the TNF-enhanced killing requires a functional H_2O_2-MPO-halide system [75]. The priming of neutrophils by TNF is reflected by an increase in expression of CR3 and CR4 on the surface of these cells. The enhanced killing of *S. aureus* was dependent on these receptors, given that this was not seen upon the addition of anti-CD11b and -CD11c monoclonal antibodies [76].

The use of TNF to enhance immunity against various microbial infections has not been considered appropriate because of the highly toxic and tissue damaging effects of TNF. In an effort to harness the anti-infective properties of TNF and exclude some of its tissue damaging properties, we synthesised short peptides representative of the TNF sequence [77]. One of these elevenmer peptides, $TNF_{70–80}$, was found to activate neutrophils and macrophages to increase microbial killing both *in vitro* and *in vivo* [77–81].

Our studies with *C. albicans* demonstrated that $TNF_{70–80}$ also protected against infections with this fungus (**Tables 1** and **2**). In the first set of experiments, the effect of administering either TNF or $TNF_{70–80}$ to mice infected with *C. albicans* was examined. The recovery of fungi from

Treatment	No. mice/group	Log CFU/g kidney (M ± SD)
PBS	23	7.3 ± 0.6
Amphotericin B	15	$2.7 \pm 2.4^{***}$
TNF (0.1 mg/kg)	29	$5.6 \pm 1.2^{***}$
$TNF_{70–90}$ (4 mg/kg)	9	$5.75 \pm 1.7^{**}$

Eight week old Balb/c mice were challenged with 5×10^5 CFU *C. albicans* intravenously. Treatment of mice commenced 24 h prior to infection, and continued with daily administration until 2 days post-infection. Mice were sacrificed on day 2 and kidney preparations plated on Sabouraud agar. The degree of infection was determined by enumeration of the number of organisms in the kidney at the time of euthanisation ($^{**}p < 0.01$, $^{***}p < 0.001$, 1-way ANOVA, SNK test). The research received approval from the Women's and Children's Hospital Animal Ethics Committee.

Table 1. The effect of TNF and $TNF_{70–80}$ on ***C. albicans*** infection in mice.

Treatment	Route	Dose (mg/kg)	Survivors 10 days post-infection
Vehicle control	IP	—	8
Cyclophosphamide	PO	30	2^{*}
$TNF_{70–80}$ + cyclophosphamide	IP	100	7^{ns}
$TNF_{70–80}$ + cyclophosphamide	IP	10	4^{ns}
$TNF_{70–80}$ + cyclophosphamide	IP	1	4^{ns}
$TNF_{70–90}$ + cyclophosphamide	IP	0.1	2^{*}
Azimexone + cyclophosphamide	IP	100	6^{ns}

Balb/c mice (10/group) were treated with 3 doses of oral (OP) cyclophosphamide (30 mg/kg) and infected with *C. albicans* as described in **Table 1**. Mice were also treated with three doses of $TNF_{70–80}$ at the schedule described in **Table 1**. Azimexone (used as a positive control) was administered intraperitoneally (IP) (n = 10 mice, $^{*}p < 0.05$, ns: not significant, one-sided Fisher's exact test). The research received approval from by Women's and Children's Hospital Animal Ethics Committee.

Table 2. Effect of $TNF_{70–80}$ on ***C. albicans*** infection in immunocompromised mice.

the kidneys of these mice was significantly lower than in non-treated control mice (**Table 1**). In the second experimental set-up, mice treated with cyclophosphamide became highly susceptible to *C. albicans* with the survival of mice dropping from 80 to 20%, 10 days after infection. If the mice had been treated with $TNF_{70–80}$, survival was increased with 70% survival observed at the highest dose (**Table 2**).

Cytokines also influence the ability of macrophages to phagocytose and kill fungi. Human monocyte-derived macrophages (MDMs) treated with interferon gamma showed increased ability to phagocytose and kill yeast forms of *C. albicans* [82]. The cytokine treated cells showed a corresponding increase in ROS production when challenged with the fungus. This effect of interferon gamma was evident with non-opsonised *C. albicans* and was independent of CR3. These effects of interferon gamma were reproduced with mouse peritoneal macrophages [83]. M-colony stimulating factor has also been shown to increase macrophage phagocytosis and killing of *C. albicans* yeast forms and cause damage to hyphae [84].

From the described studies, it is evident that when considering killing of microbial pathogens including *C. albicans*, this needs to be interpreted in terms of the cytokine milieu generated during the infection. It is evident from other published work that several cytokines regulate phagocyte-mediated microbial killing properties, including interferon gamma, lymphotoxin and interleukin-1 [71, 85].

8. Primary immunodeficiency diseases associated with susceptibility to fungal infection

Primary immunodeficiency diseases (PID) are a heterogeneous group of inborn errors of immunity. Affected individuals develop severe, unusual or recurrent infections, and may also develop features of immune dysregulation with autoimmune manifestations. There are currently over 320 described molecular genetic causes of PID, which can be categorised according to presenting phenotypic features [86]. The International Union of Immunological Sciences (IUIS) classify PID into the following disease categories: immunodeficiencies affecting cellular and humoral immunity, combined immunodeficiencies (CID) with associated or syndromic features, predominantly antibody deficiencies, diseases of immune dysregulation, congenital defects of phagocyte number, function or both, defects in intrinsic and innate immunity, auto-inflammatory disorders, complement deficiencies and phenocopies of PID [86].

Intact immunological processes and pathways are required to mount an effective immune response against fungi, incorporating both innate and adaptive components [87]. Several immune cells and immunological mediators such as cytokines are of critical importance to maintenance of anti-fungal immunity. These include phagocytes, dendritic cells, T cells (particularly T helper 1 (TH1) and T helper 17 (TH17) cells) [87]. The importance of these effectors is evidenced by patients with PID affecting cellular or phagocytic immunity developing severe, invasive or recurrent fungal infections [1].

Primary phagocytic disorders result from mutations in genes encoding key proteins that are essential for normal phagocytic development and function. These disorders may be classified

according to whether phagocyte number, function or both are affected, and by the presence or absence of associated syndromic features [86]. These disorders and their underlying, causative genetic abnormality are summarised in **Table 3**.

Congenital defects of phagocytic number, function or both			
Associated with syndromic features		**Not associated with syndromic features**	
Disorder	**Gene(s)**	**Disorder**	**Gene(s)**
Shwachman-Diamond syndrome	*SBDS, DNAJC21*	Elastase deficiency (SCN1)	*ELANE*
G6PC3 deficiency (SCN4)	*G6PC3*	Kostmann disease (HAX1 deficiency; SCN3)	*HAX1*
Glycogen storage disease type 1b	*G6PT1*	GFI1 deficiency (SCN2)	*GFI1*
Cohen syndrome	*COH1*	X-linked neutropaenia/myelodysplasia WAS GOF	*WAS*
Barth syndrome (3-methylglutaconic aciduria type II)	*TAZ*	G-CSF receptor deficiency	*CSF3R*
Clericuzio syndrome (poikiloderma with neutropaenia)	*C16ORF57 (USB1)*	Neutropaenia with combined immune deficiency	*MKL1*
VPS45 deficiency (SCN5)	*VPS45*		
P14/LAMTOR2 deficiency	*LAMTOR2*		
JAGN1 deficiency	*JAGN1*		
3-methylglutaconic aciduria	*CLPB*		
SMARCD2 deficiency	*SMARCD2*		
WDR1 deficiency	*WDR1*		
HYOU1 deficiency	*HYOU1*		
Congenital defects of phagocytic function			
Associated with syndromic features		**Not associated with syndromic features**	
Disorder	**Gene(s)**	**Disorder**	**Gene(s)**
Cystic fibrosis	*CFTR*	Chronic granulomatous disease	*CYBB, NCF1, CYBA, NCF4, NCF2*
Papillon-Lefevre syndrome	*CTSC*	Rac2 deficiency	*RAC2*
Localised juvenile periodontitis	*FPR1*	G6PD deficiency Class 1	*G6PD*
Leukocyte adhesion deficiency (LAD) 1	*ITGB2*	GATA2 deficiency (MonoMac syndrome)	*GATA2*
Leukocyte adhesion deficiency (LAD) 2	*SLC35C1*	Specific granule deficiency	*C/EBPE*
Leukocyte adhesion deficiency (LAD) 3	*FERMT3*	Pulmonary alveolar proteinosis	*CSF2RA, CSF2RB*

Adapted from [86].
SCN = severe congenital neutropaenia, WAS = Wiskott-Aldrich Syndrome, GOF = gain of function.

Table 3. Primary immunodeficiency diseases affecting phagocytic number and/or function.

Of the described primary immunodeficiency diseases of phagocytic number or function, recurrent or invasive candidal disease has been reported in cases of chronic granulomatous disease and myeloperoxidase deficiency [1] and GATA2 deficiency [88]. Candidosis is reported but tends to be less common in leukocyte adhesion deficiency and congenital neutropaenic syndromes [1].

Chronic granulomatous disease (CGD) occurs as a result of defects in components of the NADPH oxidase system, resulting in defective neutrophil oxidative burst and susceptibility to a narrow range of organisms, particularly those which are catalase-producing. As well as the predisposition to infection, patients with CGD develop a hyperinflammatory response and granuloma formation [89]. X-linked CGD occurs due to mutations in the *CYBB* gene which encodes the NADPH oxidase complex component $gp91^{phox}$ [86]. Autosomal recessive forms of CGD are less common, and occur due to mutations in the *NCF1*, *CYBA*, *NCF4* or *NCF2* genes, which encode for other components of the complex, namely $p47^{phox}$, $p22^{phox}$, $p40^{phox}$ and $p67^{phox}$, respectively [86, 89].

Candidosis is well described in CGD patients, with candidal species implicated in episodes of meningitis, fungaemia, suppurative adenitis, pneumonia, subcutaneous abscesses and liver abscess reported in a cohort of 368 patients with CGD [90]. Although the majority of these infections were expected to be due to underlying, impaired phagocytic function, additional factors such as steroid use likely increase the risk of invasive candidiasis. Candidal oesophagitis, keratitis and disseminated infection (particularly affecting young infants) have also been described, however mucocutaneous candidiasis is uncommon in CGD patients [1].

Patients with $gp40^{phox}$ mutations have been noted to have a distinct clinical phenotype as compared with those with other forms of CGD, with a milder clinical course and lower frequency of invasive fungal infection [91]. There is no impairment in the ability of the neutrophils of affected patients to kill candida, suggesting residual NADPH oxidase activity and a potential $gp40^{phox}$-independent process for reactive oxygen species production. Furthermore, monocyte and monocyte-derived macrophage NADPH oxidase generation appears to occur independently of $gp40^{phox}$ [91]. In patients with CGD, a correlation has been shown between residual production of reactive oxygen intermediates (ROI) and improved long-term survival [92]. The specific mutation in NADPH oxidase predicts the amount of residual production of ROI [92].

CGD may be conservatively managed with antibiotic and antifungal prophylaxis, along with adjunctive therapies including subcutaneous interferon therapy. CGD is curable by haematopoietic stem cell transplantation (HSCT), and trials are underway to evaluate the role of gene therapy as an alternative definitive management strategy [93].

MPO deficiency is autosomal recessive with variable penetrance, may be complete or partial, and has an estimated incidence of between 1:2000 and 1:4000 individuals [94]. Most individuals are clinically asymptomatic, although severe infections are reported in around 5% of those affected. MPO-deficient phagocytes have an impaired capacity to kill *C. albicans*, as evidenced by severe infection in MPO-deficient mice.

GATA2 encodes a zinc finger transcription factor which is critical for haematopoetic cell development [95]. Mutations in this gene give rise to a syndrome also known as 'MonoMac', which

refers to the monocytopaenia and predisposition to mycobacterial infection which are characteristic of this condition [95, 96]. In addition, affected patients have other haematological anomalies including thrombocytopaenia and neutropaenia, predisposition to haematological malignancy and severe mycobacterial, fungal and human papilloma viral infections [88, 96]. In a recent study of 79 French and Belgian patients with *GATA2* mutations, 16 patients were reported to have had 18 episodes of fungal infection, 5 of which were candidoses [88]. Eight of the 18 infections were associated with chemotherapy or HSCT. The neutrophils from some *GATA2* deficient patients were noted to have reduced granularity [97]. When stimulated with PHA (phytohaemagglutinin), patient PBMCs (peripheral blood mononuclear cells) demonstrated reduced lymphocyte proliferative and cytokine production capacity, which normalised after addition of monocytes [96], highlighting the important role of these cells in eliciting an effective immune response.

In addition to the critical role of phagocytes in anti-fungal immunity, defects in other immune cells and immunologic pathways also give rise to susceptibility to infection with candida and other fungi. A range of single-gene inborn errors of immunity resulting in severe or recurrent superficial or invasive candidiasis have been described [86, 98]. Cell-mediated immunity is essential for anti-fungal immunity. This is evidenced by the predisposition to severe fungal infection in infants with severe combined immunodeficiency (SCID), a life-threatening condition manifested by low, absent or severely dysfunctional T cells [86]. Other forms of combined immunodeficiency, for example, those due to deficiencies in CD25, NEMO/IKBG, DOCK8, TCR-α, ORAI1, MST1/STK4, MHC Class II, along with *IKBA* gain of function mutations and idiopathic CD4+ T cell lymphopaenia are associated with chronic mucocutaneous candidiasis (CMC) [98]. In addition, CMC is a feature of several PID with syndromic features, including STAT3 deficiency (autosomal dominant hyper-immunoglobulin E syndrome), APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dysplasia), also known as APS-1 (autoimmune polyglandular syndrome type 1) which occurs due to mutations in the *AIRE* gene), and deficiencies of IL12Rβ, IL-12p40 and CARD9 [98, 99]. The importance of the TH17 pathway and IL-17 signalling in anti-candidal immunity has become apparent [100, 101], with severe CMC described in patients with deficiencies of IL-17RA, IL-17F, RORC and *STAT1* gain of function mutations [98, 102]. In particular, AIRE has been demonstrated to have a key role in anti-candidal immunity, as evidenced by its role in fungal synapse formation which is required for initial macrophage recognition of fungal hyphae [103]. AIRE, along with Dectin-1, Dectin-2, Syk and CARD9 are required for formation of the fungal synapse upon stimulation of macrophage-like THP-1 cells after stimulation with *C. albicans* [103].

9. Secondary immunodeficiency diseases associated with disorders of phagocyte number or function

Immunosuppression is a well-described risk factor for infection with candida and other fungal species [98]. Corticosteroids are commonly used in the management of a range of inflammatory and malignant conditions, and use of these agents is a known risk factor for fungal infection [104]. The precise mechanisms by which corticosteroids lead to impaired anti-candidal

immunity remain unclear, and this is likely multifactorial [105]. In terms of phagocytic cell function, corticosteroids appear to alter leukocyte differentiation programs. They induce monocytes and macrophages to adopt an anti-inflammatory phenotype. This is modulated by the cytokine environment (including increased IL-10 expression on macrophages), increased apoptotic activity and induction of transcription of anti-inflammatory genes which impact upon chemotaxis, phagocytosis and resistance to oxidative stress [105]. However, despite these observations it has been recently shown that dexamethasone increases the expression of CRIg on human MDMs but not CR3 or CR4, and that this increase was associated with an increase in phagocytosis of complement opsonised *C. albicans* [23, 26, 27].

Cancer patients are at an increased risk of systemic candidiasis, and *C. albicans* is reported to be one of the most common causes of sepsis in this patient group [104]. This predisposition to fungal infection is multifactorial, and may be due to a secondary immunodeficiency caused by the underlying malignancy itself, or due to the effects of chemotherapeutic agents. Chemotherapeutic drugs may induce neutropaenia or affect neutrophil function, thereby impairing anti-candidal immunity. Neutrophil function may be impaired as a result of reduced trafficking, chemotaxis or phagocytic activity. For example, chemotherapeutics targeting microtubule structures likely impair cytoskeletal processes and actin polymerisation, thereby reducing neutrophil chemotaxis and phagocytosis. Chemotherapeutic agents can also interfere in NETosis, which is important for antimicrobial activity. Some drugs may also induce monocytopaenia and impaired monocytic function, further increasing the risk of candidal infection [104].

Patients with liver disease are at an increased risk of fungal infection. Those with cirrhosis have been found to have reduced complement levels and impaired monocyte activation and neutrophil mobilisation [106]. Patients with liver disease are at risk for infectious peritonitis, and *C. albicans* and *C. neoformans* were amongst the main species isolated in these cases. Renal disease is also a risk factor for invasive fungal disease [104]. Neonatal candidal sepsis has been reported in association with jaundice [107]. Interestingly, unconjugated bilirubin in hyperbilirubinemia has also been linked to reduced phagocytic cell function; phagocytosis and killing of fungi [108, 109]. Burns patients are at increased risk of fungal infection owing to a breached skin barrier and use of antimicrobial agents, with candidal infection in particular being associated with increased morbidity and mortality in these patients [106]. In addition to these disease states, other physical factors, alone or in combination, such as the use of intravenous catheters and mechanical ventilation also increase the risk of invasive fungal disease [98, 104].

Finally, it is also evident that anti-fungal drugs *per se* can compromise immunity [109–111]. Several of the imidazoles were found to inhibit neutrophil functions, chemotaxis, phagocytosis and microbial killing of bacteria and candida [110].

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Conflicts of interest

Authors AGS and JRK declare no conflicts of interest. Authors DAR and AF declare that they are inventors on patent relating to $TNF_{70–80}$ technology.

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Chapter 6

Interactions of *Candida albicans* Cells with Aerobic and Anaerobic Bacteria during Formation of Mixed Biofilms in the Oral Cavity

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Additional information is available at the end of the chapter

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Abstract

Biofilm is a compact coating formed on various artificial and physiologic surfaces by a population of microorganisms which in this habitat establish a close cooperation, exploiting both the physical interactions that stabilize the community and chemical cooperation, engaging numerous agents to modify the environment, i.e., to influence the acidity, nutrient acquisition, or oxygen availability. Microorganisms can also communicate using quorum-sensing molecules carrying specific messages. Some microbes temporarily dominate, while others are constantly replaced by different community members. But these co-operations or competitions have a deep sense—they serve to protect the whole community against the defense system of the host to assure survival. The oral cavity is inhabited by diverse microorganisms, including bacteria, but also yeast-like fungi from the genus *Candida* that stay under a tight control of the host as long as its immune system is not weakened; then these relatively mild commensals convert to dangerous pathogens that start the invasion often in collaboration with other microbes. Elongated hyphal forms of fungal cells favor the biofilm type of growth and communication with other microbes supporting immune resistance of the biofilm. In this chapter, we discuss the mechanisms of interactions between bacteria and *C. albicans* in the oral cavity, their communication, host responses, and possible strategies of anti-biofilm treatment.

Keywords: *Candida albicans*, biofilm, aerobic and anaerobic bacteria, quorum-sensing, host responses, anti-biofilm therapies

1. The oral cavity: a common place for polymicrobial biofilm formation

The oral cavity comprises the most complex niches of the human body colonized by a wide variety of bacteria and fungi species. These commensal or often opportunistic and pathogenic colonizers tend to form biofilms—structured microbial communities, attached to natural or artificial surfaces, which are directly attributable to the virulence of these microorganisms and their ability to cause infections [1].

The variety of microbial species inhabiting the oral cavity results from the presence of two different functional surfaces, the mucosal surface and the teeth, representing various conditions in terms of nutrient and oxygen availability [2]. The microorganisms that early colonize the salivary pellicle on the tooth surface are streptococci such as the oral commensal—*Streptococcus gordonii.* Further biofilm formation involves some bridging microorganisms such as *Fusobacterium nucleatum* [3]. As the biofilm extends below the gum line and becomes subgingival plaque, more pathogenic, Gram-negative anaerobes such as *Porphyromonas gingivalis* or *Tannerella forsythia* are embedded [4].

For polymicrobial growth and survival in the human oral cavity, establishing a well-functioning community, i.e., biofilm, is essential. The formation of biofilm increases a resistance to antimicrobial agents and nutritional changes. However, the transition from planktonic type of growth to biofilm community requires many transcriptional and proteomic changes. Most of them concern co-aggregation/-adhesion processes, sensing diffusible signals, and metabolic interactions. Such a developed biofilm is still exposed to changes of nutrition and oxygen availability, pH fluctuation, antimicrobial properties of saliva, and is also modified by the contact with host tissues [5].

The latest studies of oral microbiome pointed at an opportunistic inhabitant of oral mucosa—the *Candida albicans* yeast-like fungus—as an important biofilm player among microbiota that contacts with mucosal tissues of the host. Under colonization or infection conditions, *C. albicans* adheres to tissues, interacting with a variety of host extracellular matrix molecules that promote adhesion to the host surfaces [6]. The adherence is strictly dependent on *C. albicans* ability to switch morphology between yeast and hyphal forms [7, 8].

Numerous observations supported a hypothesis that fungi have a beneficial or favorable role in maintaining the healthy balance between microbes and the host. On the other hand, *C. albicans* well adapted to constantly changing demands in the human host environment [9] seems to be able to use the different colonizing strategy under situations that emerge in the pathological disparities.

Tracking the yeast oral infections showed that the same initiating and bridging microorganisms composing biofilms were involved in the interaction with hyphal filaments of *C. albicans*, promoting co-colonization of these surfaces by yeast [10]. Moreover, the interactions between yeast and streptococci appear to be synergistic. In addition to providing adhesion sites, streptococci excrete lactate that can act as a carbon source for yeast growth [11]. On the other hand, *C. albicans* may provide bacteria with growth stimulatory factors, resulting from the nutrition metabolism [12] and can reduce the oxygen pressure to the level, preferred by streptococci.

C. albicans also co-aggregates with an obligatory anaerobe, *F. nucleatum* [13], or a facultative anaerobe—*Actinomyces oris* [14].

Current studies [15–17] report that *C. albicans* biofilms protect the obligatory anaerobes, like *P. gingivalis* and *T. forsythia*, under aerobic culture conditions. It is possible because oxygen depletion within the structured fungal biofilm or its fast consumption by fungal cells results in creation of anaerobic micro-niches that help strict anaerobic bacteria to survive and proliferate. The observed depletion of oxygen could depend on the number of *C. albicans* cells or their respiratory rate.

2. Mechanisms and structures involved in formation of *Candida albicans* biofilms

The ability of *C. albicans* to form a biofilm is closely related to the virulence potential of pathogenic form of *C. albicans* and is characterized by a high heterogeneity among different clinical isolates [18, 19]. This form of fungal community depends on the cell surrounding and proceeds via sequential steps. The process starts with the initial adhesion of single yeast-like cells to the artificial or mucosal surface (**Figure 1**) and formation of a basal yeast cells layer [20–22]. During this phase both the surface properties on which microorganism form aggregate, its structure, charge, hydrophobicity, or roughness, as well as the structure of molecules present at the surface of pathogen cells play important roles [23]. In the further cell proliferation step, the fungi develop the filamentous hyphal form of the cells accompanied by production of an extracellular polysaccharide matrix in mature biofilm, which protects them, and strengthens the biofilm structure [24]. These processes lead to a significant increase in the thickness of the biofilm, and its maturation is controlled by at least nine master transcription regulators (Ndt80, Bcr1, Efg1, Rfx2, Flo8, Rob1, Brg1, Gal4, and Tec1) that supervise the network of about 1000 of targeted genes involved in biofilm formation [21, 25]. Then, the dispersion of biofilm-associated yeast-like cells can occur with further fungal cell dissemination, often associated with invasive diseases [24, 26].

Numerous different mechanisms and molecules are involved in the overall complex process of *C. albicans* biofilm formation [27]. Initially, the general physicochemical properties of *C. albicans* cell surface and subsequent activity of cell wall adhesive proteins play extremely important roles, allowing the cells to adhere to the targeted substrates or materials [28]. This essential group of molecules responsible for in vitro and in vivo biofilm development includes several proteins covalently bound to the fungal cell wall and equipped with a signal peptide for classical secretion and glycosylphosphatidylinositol (GPI)-anchor site, i.e., hyphal cell wall protein Hwp1 [29], proteins from agglutinin-like sequence (Als) protein family, such as Als1 and Als3 [28], and hyphally regulated cell wall protein Hyr1 [28]. Their transcription is regulated by the transcription factor Bcr1 [30] and is primarily associated with the morphological transition from yeast cells to filamentous forms, thus implicating their association mainly with the cell wall of hyphae [31–33]. Additionally, other adhesins are required for *C. albicans* adhesion and proper biofilm formation, including Eap1 (enhanced adhesion to polystyrene 1) protein present at the cell surface of both yeast cells and hyphal forms [34, 35]. Adhesion-related proteins are important not only for the binding of fungal cells to the receptors on host

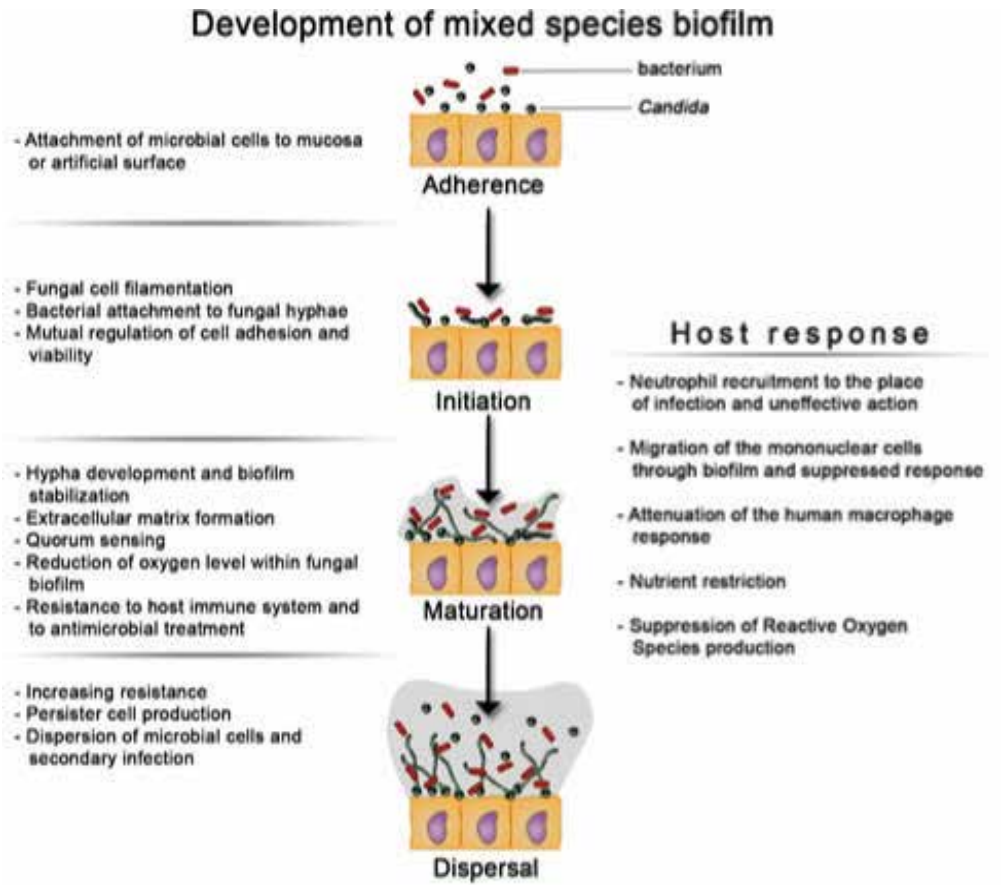

Figure 1. Polymicrobial biofilm: stages of development and host responses.

tissues or to the artificial surfaces, but also for maintaining the cell-cell interactions within the biofilm that allow further stabilization of the structure and avoiding the removal of fungal cells by the action of host defense mechanisms such as the salivary flow. In the process of aggregation and intracellular interactions between fungal cells, fragments of adhesins that consist of amino acid sequences predicted to form amyloid-like β-aggregates and mediating amyloid formation may participate, as described for the protein Rbt1 (repressed by TUP1), a GPI-anchored cell wall protein with a similarity to Hwp1 [36, 37].

After the adhesion step, further proliferation of cells and production of filamentous forms lead to the enhanced development of biofilm [38], processes related not only to the change in the surface properties of fungal cells and the increase of their adhesiveness, but also to the production of further virulence factors and biofilm matrix components [24]. Among the large repertoire of extracellular hydrolytic enzymes produced by *C. albicans* that play a pivotal role during the invasion on host tissues during the infection and are involved in biofilm-related pathogenesis, representatives of families of lipases, phospholipases, and secreted aspartyl proteinases (Saps) can be included [39]. The major biofilm-associated Saps are hypha-specific Sap5

and Sap6, responsible for the acquisition of nutrients, aggregation of fungal cells, intracellular communication, and production of extracellular matrix during biofilm development [40–42].

The highly complicated and heterogeneous extracellular biofilm matrix (ECM) is composed of numerous proteins (55%), carbohydrates (25%), mainly branched α-1,6-mannans, unbranched β-1,6-glucans, and β-1,3-glucans, as well as lipids (15%), including neutral glycerolipids, polar glycerolipids and sphingolipids, and nucleic acids (5%) [43]. The matrix that strengthens the biofilm significantly contributes to the development of biofilm resistance to adverse environmental conditions and penetration of antifungal drugs [43–45]. Several matrix-associated proteins have been identified, both involved in the basic cellular metabolism, as well as the proteins responsible for the rearrangement of the matrix structure and maintenance of its functionality (glucan-modifying enzymes and protein mannosyltransferases, i.e., Xog1, Exg1, Bgl2, Pmt1, Pmt2, Pmt4, Pmt6) [46, 47]. Extracellular DNA (eDNA) detected in *C. albicans* biofilm matrix is probably mainly responsible for the structural integrity [48].

In the process of biofilm dispersion, the molecular chaperone, heat shock protein 90 (Hsp90) is strongly involved [49], affecting the morphogenetic transition from yeast cells to hyphal forms and repressing Ras1/PKA (cAMP-dependent protein kinase) signaling cascade [50]. Furthermore, the conserved histone deacetylase complex, including Set3, Hos2, Snt1, and Sif2 proteins also participates in the dispersal of biofilm, modulating the transcription kinetics of the genes that regulate biofilm maturation [51].

3. *C. albicans* interactions with bacteria during mutual biofilm formation

C. albicans colonizes the oral cavity, presenting the commensal or pathogenic properties that can be modified by direct or indirect interactions with different types of bacteria, depending on the localization of the microbial communities, such as the supragingival plaque, subgingival plaque, and tongue coating. The metabolic activity of microorganisms that colonize the supragingival sites, i.e., nonmutans streptococci and *Actinomyces* enriches the environment in lactic acid, creating a temporarily acidic environment that favors the entrance of the more cariogenic microorganisms, mutans streptococci into the ecosystem. Conversely, at subgingival sites, colonized by *Fusobacteria* and *Prevotella*, a neutral pH and anaerobic environment dominate and facilitate the establishment of a less acid-tolerant but periodontopathogenic bacterium, *P. gingivalis* [52].

The mitis group of streptococci (MGS), including *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus gordonii*, and *Streptococcus sangunis* [53, 54] belongs to the early colonizers of oral cavity and appears to interact synergistically with *C. albicans* hyphal filaments providing the physical and metabolic interactions by the exposition of specific adhesion sites and excreting lactate that serves as a carbon source for yeast growth [55]. On the other hand, *C. albicans* can provide bacteria with growth stimulatory factors, resulting from the fungal nutrition metabolism [12] and reduces the oxygen pressure to the level, preferred by streptococci. Moreover, a mutual collaboration of *C. albicans* and *S. oralis* increases the inflammatory host responses

compared to monospecies infections [56, 57]. The consequence for the host is provided by the precise interactions between the cell surface proteins of *S. oralis*, *S. mitis* and *S. gordonii*, especially SspA and SspB [58, 59] and the candidal cell wall adhesins of agglutinin-like family, mainly Als3, presented on the surface of fungal hyphae [60]. Some parts of these proteins seem to be particularly important for the interactions but a precise mechanism of the interaction requires further research, especially to clarify a significance of cell wall mannosylation [57, 61] in this process.

Moreover, the polysaccharides of both types of interacting microorganisms that compose ECM not only protect the cells, but also create a new platform for mutual interactions between fungal glucans and mannans and bacterial glucosyltransferases within the ECM matrix structure [46, 62, 63].

The best example of microbial cooperation for increased pathogenic properties of mixed biofilm is represented by the interactions of *C. albicans* with *S. aureus*, identified in oral cavity and being a source of systemic infection [64–67]. The bacteria prefer hyphal filaments of *C. albicans* [68, 69] for adhesion, but its localization within the biofilm seems to depend on the surface colonization sequence. When bacteria are the first colonizer, the development of fungal biofilm is slower and bacteria cells are spread in whole three-dimensional biofilm structure [68]. On the other hand, a simultaneous contact of microorganisms with the surface favors the rapid formation of the mixed biofilm with *S. aureus* localization within upper layers of fungal biofilm and with involvement of multiple microbial proteins. However, the role of fungal adhesins from Als family in these interactions has been questioned [70]. The formed biofilm and its ECM with extracellular fungal DNA protect the bacteria against the antibiotic treatment [71–73].

C. albicans co-aggregates with an obligatory anaerobe *F. nucleatum* [13], with engagement of a mannan receptor on the *C. albicans* surface [74]. A facultative anaerobe—*A. oris*—makes its own carbohydrate-containing surface molecules available to the interaction with *C. albicans* [14]. Last studies have also shown that *C. albicans* is able to interact with keystone pathogen of subgingival plaque—*P. gingivalis*—the obligatory anaerobe. However, it is difficult to judge whether the pathogens apply a synergistic or concurrence style of interactions. It was demonstrated that *P. gingivalis* suppressed *Candida* biofilm formation by a reduction of fungal cell viability [75]. Recently, the gingipain activity has been suggested to be the main destructive force influencing fungal cells wall within the mixed bacterial-fungal biofilm (unpublished data). On the other hand, *P. gingivalis* was also shown to induce germ-tube formation by *C. albicans* cells, generating a more invasive phenotype of fungal cells [76]. But such an effect could also result from fungal protection toward contacting bacteria and their virulence potential (unpublished data). The mutual interactions are supported by the involvement of adhesins, especially fungal Als3, Mp65 and surface-located enolase, aand a bacterial internalin, InlJ or gingipains ([77], unpublished data). The important role in the interaction between *C. albicans* and *P. gingivalis* has been also assigned to peptydylarginine transferase of *P. gingivalis* (PPAD), the enzyme capable of modifying Arg residues to citrullines. Its action can directly contribute to the change in the spatial structure of the molecule [78]. A bacterial mutant deprived of PPAD forms a reduced mixed biofilm compared to the wild-type strain. Potential molecules whose citrullination may affect the effectiveness of biofilm formation include arginine-specific cysteine proteinase (RgpA) and adhesive Mfa1 fimbrilin [17, 79].

The interaction of both microbes seems to exert marked consequences to the host. However, it was presented that both microbes appear to have antagonistic effects on one another, as *P. gingivalis* inhibited the adhesion of *C. albicans* to buccal epithelial cells [80]. But the presence of *C. albicans* did not enhance adhesion of *P. gingivalis* to gingival epithelial cells or gingival fibroblasts. On the other hand, a pre-exposure of gingival epithelial cells and fibroblasts to *C. albicans* enhanced the cell invasion by *P. gingivalis* [81].

4. Host responses to the candidal biofilms

Clinical candidal oral biofilm inhabiting mucosal surfaces or artificial devices may trigger more or less similar host responses (**Figure 1**). Regardless of the biofilm origin, it remains under the influence of immune factors produced by contacting epithelial cells [82].

Dongari-Bagtzoglou et al. [83] analyzed the candidal biofilm in a murine model and found that this fungal cell community induced a hyperkeratotic response and epithelial cell desquamation. Moreover, the matrix that surrounded the fungal biofilm was enriched in keratin and desquamated cells.

Also, in a rat model of chronic denture gingival dermatitis, the host proteins were prominent in the extracellular matrix, including amylase, hemoglobin, and antimicrobial peptides [84, 85].

The oral biofilm elicits responses of human immune cells (**Figure 1**). The neutrophil migration and their deeper localization within the biofilm were identified, but these defense cells were not effective in clearing these infections [82]. An analysis of neutrophil responses in the ex vivo models of their contact with *C. albicans* biofilm also showed a diminished activity of neutrophils against this structured fungal community, compared to the responses against the planktonic form of fungal cells [86].

Upon a contact with pathogens, neutrophils can activate many mechanisms of response to suppress the infection. These include degranulation, phagocytosis, and neutrophil extracellular traps (NETs) formation [87]. The latter process aims at entrapment of large objects such as fungal hyphae [88]. Nevertheless, a group of Johnson showed that neutrophils failed to release NETs in contact with fungal biofilm [89]. These results were described as *an immunological silence*, where host immune system ignored contacting biofilm because of its shielding by the matrix components [90]. The biofilm matrix prevents the exposition of so called pathogen-associated molecular patterns (PAMPs) that can be recognized by highly specialized pattern recognition receptors (PRRs) of human immune cells [91, 92].

Another explanation proposed to interpret the evasion of host response by the biofilm was *an immune deviation* that could result from action of yet unidentified fungal compounds. They could act directly or indirectly by triggering host immunomodulatory factors that transform the immune response into ineffective form [93]. Such hypothesis was supported by the observation that *C. albicans* cell wall components were able to induce the expression of Il-10 influencing Th2 response [94].

A further explanation of biofilm survival was represented by a model of *immune resistance* proposed in [95], where GPI-anchored cell wall protein Hyr1 could play an important role

[96]. Moreover, all of proposed mechanisms or their combinations could be involved in local paucity of PMN responses. Katragkou et al. [86, 97] and Xie et al. [98] documented that developed biofilm covered by ECM exposed fungal β-glucans that were involved in hindered neutrophil responses to cytokine priming of PMNs or fungal cell opsonization. Such an argument was also strengthened by the observation that pre-treatment of PMNs with interferon-γ or granulocyte colony-stimulating factor (G-CSF) did not significantly enhance their activity against opsonized or nonopsonized *C. albicans* biofilms. Moreover, neutrophils contacting the mature biofilm did not produce reactive oxygen species, necessary for triggering of phagocytosis, or one of the pathways of NET production [99]. Nevertheless, the precise mechanism of this phenomenon remained to be clarified.

Similar, diminished responses were also observed for a contact of fungal biofilm with mononuclear cells, compared to their co-culture with fungal planktonic form [100]. Although the migration of the mononuclear cells through biofilm was detected with their main compaction in the basal part of biofilm, their phagocytosis properties were suppressed, and the production of pro-inflammatory cytokines in response to biofilm decreased. Surprisingly, the mononuclear cells augmented biofilm proliferation, increasing the biofilm thickness over two-fold [97, 101].

Most of the presented observations were made concerning host response to contact with fungal biofilm, but the host immune system usually has to face an ongoing polymicrobial infection [102] about which the information are rather scarce [103]. An example of the cross-kingdom infection of the human host was represented by *C. albicans* biofilm contacting gingival anaerobic bacteria, *P. gingivalis.* In this case, an attenuation of the human macrophage responses was observed [17]. Moreover, some studies presented that the host responses can vary depending on the pathogen that contacts the fungal biofilm [104]. The pathogen interactions can be synergistic as well as antagonistic. For example, in a rat model, the colonization of the airway by *C. albicans* impaired functions of alveolar macrophages and, in consequence, led to the reduced clearance of *Pseudomonas aeruginosa* [105]. On the other hand, Lopez-Medina et al. [106] showed in a mouse gut model that the co-infection of *P. aeruginosa* with *Candida* cells suppressed the expression of bacterial genes responsible for iron acquisition, and thus suppressed the infection. Nevertheless, our understanding of host responses to mixed biofilm formed between different type of pathogens remains still at its infancy and needs many further studies.

5. Quorum sensing within the mixed biofilm and its significance for the host

An important phenomenon occurring in the process of biofilm formation is also the transmission of signals between microbial cells located within the biofilm, thus stimulating them to further growth and dispersion of the cells or, in contrary, suppressing them. In addition, signaling molecules can also affect microbial cells of other species that inhabit the same niche in the host organism, and thus promote synergistic or antagonistic interactions between different pathogens which can result in clinical outcomes. This phenomenon of the communication between microorganisms through the secretion of low molecular weight compounds, referred

to as the quorum sensing (QS) [107], involves specific chemical compounds whose increasing concentration is a signal to change the expression of selected genes in the cells of the entire biofilm population [108].

C. albicans produces autoregulatory substances involved in quorum sensing (quorum-sensing molecules, QSMs) that affect important virulence traits, such as transformation of the morphological forms [109]. One of them is farnesol—an alcohol from the terpene group, secreted by *C. albicans* in the later stages of biofilm formation, with a function of blocking the formation of filamentous forms of this yeast [110]. A function opposite to farnesol has a second fungal QS compound, tyrosol, which stimulates the phase of active growth of the *C. albicans* cell population and the formation of hyphae in the initial phases of biofilm formation, thus increasing the thickness of the biofilm [111–113].

When the concentration of farnesol is higher than that of tyrosol, the conversion of yeast form to hyphae is inhibited and a release of individual cells from the biofilm is stimulated. Such effect indicates possible interactions between these two QS systems in the process of biofilm building [112]. Additionally, *C. albicans* secretes two aromatic alcohols, phenylethyl alcohol and tryptophol, also identified as QSM [113].

The role of QSM seems to be particularly important in mixed biofilms, in which the coexistence of fungi and bacteria is associated with their mutual communications. QSM secreted by the bacteria can exert both stimulatory and inhibitory effects on the cell morphology and biofilm formation by *C. albicans* cells. It is likely that a combination of these contradictory signals orchestrates the balance between the cellular and filamentous form in biofilms, preventing the excessive growth of *C. albicans* within these communities [114].

One example of cross-species communication using QS signals are biofilms formed between *C. albicans* and Gram-negative bacteria *P. aeruginosa*. It has been shown that the presence of farnesol produced by *C. albicans* inhibits functioning of bacteria, and suppresses the production of a bacterial quinolone signaling molecule—PQS, and the piocyjanin—an important bacterial virulence factor [115]. On the other hand, under formation of mixed biofilms, *P. aeruginosa* produces homoserine lactone that may fulfill a role similar to farnesol, reducing the production of fungal hyphae *in vitro* [116].

Another example is the biofilm with participation of *S. mutans*, in which the inhibition of biofilm formation was observed in response to a high concentration of farnesol (>100 μM), while a low level of farnesol (~25 μM) promoted bacterial growth [117–119].

In other studies, *S. mutans* could both, reduce the farnesol production by *C. albicans* [119] and inhibit the formation of filamentous form of *C. albicans* by the competence-peptide CSP, produced on the early stages of biofilm development [120, 121].

The same peptide produced by *S. gordonii* inhibited the formation of *C. albicans* biofilm, but not the hyphal growth [122]. In contrast, other bacterial QSM—the autoinducer-2 (AI-2), as well as H_2O_2 secreted by *S. gordonii* affected the morphogenesis and production of farnesol. The strains with the deletion of the LuxS quorum-sensing system responsible for AI-2 production in *S. gordonii* presented a reduced ability to stimulate the growth of *C. albicans* hyphae

and thereby a general reduction of biofilm biomass. The identified responses correlated with an invasion into the host epithelial cells [10, 27].

An interesting interspecies communication is presented by the Gram-negative *Aggregatibacter actinomycetemcomitans*, acting in periodontal disease, which can inhibit the formation of *C. albicans* biofilm by producing AI-2. Although AI-2 has been described as QSM of different bacteria, other species give off other AI derivatives, so that the results obtained for different species do not have to be identical to one another. Interestingly, *A. actinomycetemcomitans* is one of the bacterium having a dual inhibitory system acting toward *C. albicans* biofilm. In addition to QSM, it also includes cytolethal distending toxin (CDT). One of the emerging hypotheses suggests that secreted QSM is a warning signal for *C. albicans* against a competitor that secretes the toxins [120, 123].

QSM also plays an important role in a communication between *C. albicans* and the Gram-positive bacterium—*S. aureus*. Farnesol, secreted by *C. albicans* inhibits the formation of *S. aureus* biofilm and increases its susceptibility to antibiotics [124, 125]. There were also studies, indicating that *S. aureus* stimulated the growth of *C. albicans* biofilm possibly by QSM [69]. It was also proposed that in the presence of farnesol, *S. aureus* acquires a resistant phenotype that induces oxidative stress, resulting in the upregulation of bacterial drug efflux pumps [126].

QS production within the biofilm has also an impact on the efficiency and the functioning of host defense systems. The gingival epithelial cells presented an upregulation of the toll-like receptor TLR2, and a decrease of the expression of TLR4 and TLR6 upon treatment with farnesol, suggesting the resulting activation of antifungal defense. Considering the role of epithelial cells in the secretion of pro-inflammatory cytokines, it was also shown that farnesol increased the secretion of IL-6 and IL-8. Moreover, farnesol modulated the secretion of antimicrobial peptides by epithelial cells, including hBD1 and hBD2. *C. albicans* cells, via production of farnesol, suppressed the epithelial secretion of hBD1, with a simultaneous increase in hBD2 secretion. Since both peptides have a high efficacy in *C. albicans* killing, the results suggest that farnesol may be a key factor in promoting host defense [127]. An additional function performed by farnesol includes its ability to activate neutrophils and monocytes and to reduce the phagocytic activity of mouse macrophages. Farnesol also impairs the differentiation of monocytes into dendritic cells and decreases their ability to activate and expand T cells, which consequently reduce the induction of IL-12 [128].

In summary, mutual QS interactions between fungi and bacteria may play an important role as a virulence mechanism that mediates the communication between the host and the formed biofilm, and could inspire future applications in diagnostics and biofilm treatment.

6. Resistance of oral biofilm

The biofilm formed on mucosal or artificial surfaces in oral cavity is difficult to eliminate since the biofilm structure protects the pathogenic cells against antimicrobial drugs, especially against antifungal agents, and suppresses immune responses [129]. Moreover, cooperating invaders often present increasing virulence resulting from synergistic and complex

interactions between microorganisms [130]. It has been demonstrated that *Staphylococcus* adheres to yeast and hyphal forms, and this interaction benefits the growth and antibiotic resistance of *S. epidermidis*. In addition, the components of the biofilm extracellular matrix produced by the wild-type of *S. epidermidis* prevent the effective penetration of antimycotic molecules such as fluconazole into the biofilm and promote the spread of yeast infection [131].

The low susceptibility of biofilms to medical treatment is attributed to multifactorial events, represented by upregulation of efflux pumps, the presence of extracellular matrix and appearance of recalcitrant persister cells [132].

The two classes of fungal efflux pumps (FEP: Cdr1, Cdr2, and Mdr1) are activated in planktonic cells in contact with antifungal drugs but in biofilms FEP are upregulated probably in response to contact with other partners that compose the biofilm. Such an explanation was supported by an observation that FEP efficient function appeared shortly after the cell surface adhesion and remains upregulated during whole process of mixed biofilm formation [21].

Another contributor to mixed biofilm resistance is the extracellular matrix and its components. This three-dimensional complex structure effectively inhibits antibiotic and antimycotic diffusion [133]. Moreover, the biofilm-composing polysaccharides not only mask the biofilm against its recognition by the host receptors, but also can directly bind and inactivate the drugs, as it was presented in a case of antifungal-acting amphotericin B, sequestered by β-1,3-glucan, composing ECM [134].

Also, eDNA is an especially important biofilm component, whose viscosity and negative electric charge influences the structural integrity and stability of biofilms but also contributes to drug resistance via acting as drug chelator. eDNA also binds magnesium ions, whose decreased level serves as a signal, inducing PhoPQ and PmrAB systems, responsible for *P. aeruginosa* resistance to antimicrobial peptides and to aminoglycosides [135].

An important phenomenon that plays a key role in the development of drug resistance by oral microbiome is the horizontal gen transfer (HGT) [131]. The biofilm structure provides a suitable environment for gene exchange, because the microbial cells are in close proximity and the virulence genes are dynamically spread between different species of bacteria composing biofilm. The most popular mobile genetic element in oral microflora is the conjugative transposon *Tn*916, which contains genes encoding ribosomal protection proteins [131]. These proteins inhibit the action of tetracycline, the most popular antibiotic used in periodontal disease treatment, by preventing the binding of this antibiotic to the bacterial ribosome [136]. Another biofilm protective function is carried out by membrane vesicles (MVs), present in ECM [137], which protect bacteria against some antibiotics by the degradative properties of MV enzymes, such as β-lactamase [138].

The important factors that contribute to the biofilm resistance are the persister cells detected in bacterial and fungal biofilm [20, 139]. The persister cells are a minor subset of metabolically dormant cells presented within biofilms that possess extreme resistance to antimicrobial agents and are responsible for the severe chronic infectious disease. However, the mechanism of this resistance of persister cells remains to be discover; they could possibly be a good target for further antimicrobial therapies.

7. The challenges for medical treatment of mixed oral biofilm

As no biofilm-specific drugs exist today, the treatment of infections caused by mixed species community remains a major challenge for contemporary medical biotechnology and the developing of new effective strategies for biofilm eradication becomes critical.

One of the strategies for combating biofilms formed by bacteria and yeasts can be a degradation of ECM. It has been demonstrated that enzymatic degradation of some biofilm-forming components facilitates the penetration of antibiotic and antimycotic molecules and affects the biofilm structural integrity [140, 141]. For example, a study demonstrated that a combined use of deoxyribonuclease and amphotericin B reduced the survival of *C. albicans* cells.

An effective alternative to antibiotic therapy may be a treatment with anti-biofilm peptides. These compounds easily penetrate the structure of multispecies biofilm and inhibit the growth of Gram-positive and Gram-negative bacteria. An example of such an anti-biofilm compound is a short synthetic peptide 1018 (amino acid sequence: VRLIVAVRIWRR), which blocks a stress response through an activation of the stress-signaling nucleotide degradation [142]. Another example of an anti-biofilm compound is D-enantiomeric peptide DJK-5 that has a similar mechanism of action to peptide 1018 [143]. The main advantage of the DJK-5 is its resistance to proteases produced by the host and bacteria. Moreover, DJK-5 possesses a higher biological activity than peptide 1018 and kills most of the oral biofilm-forming bacteria in a few minutes. It has been demonstrated that the use of anti-biofilm peptides in combination with conventional antibiotics both increases the effectiveness of treatment and reduces the required concentration of antibiotics [144].

Several natural products have been also proposed for fungal biofilm treatment. An example of plant metabolites with antifungal activity are terpenoids, such as xanthorrhizol extracted from *Curcuma xanthorrhiza* [145]. It has been demonstrated that this compound effectively inhibits the development of mature biofilms formed by various *Candida* species. Moreover, in contrast to commonly used antifungal drugs, xanthorrhizol is nontoxic to human cells even at very high concentrations.

Also, chemical signal molecules involved in quorum sensing possess a potential for the therapy of oral infections disease. There are two main mechanisms of action of the known QS inhibitors [146]. Some of these cause an enzymatic degradation of signaling molecules. The enzymes—AHL-lactonases and AHL-acylase can be classified to this group. Other inhibitors such as furanones that are produced by red marine algae are structural analogs that prevent bacterial biofilm development via binding to LuxR [147]. In the case of oral *C. albicans* infections, the use of farnesol has been proposed [148]. In vivo studies have shown that the addition of farnesol suppresses the hyphal growth on the mouse tongue at the first step of biofilm formation, and as a result prevents the invasion of mucosal membrane by the yeast and bacteria.

An interesting proposal for the treatment of mixed biofilm can be the photodynamic antimicrobial chemotherapy (PACT) that applies the nontoxic dye (photosensitizer) activated by visible light [149]. Singlet oxygen, which is effectively produced during this process, effectively kills pathogen cells. This novel method has been successfully used against *C. albicans* biofilm

and can be a promising antimicrobial therapy that has many advantages such as the high target specificity. What is more, the development of resistance to PACT is unlikely because microorganisms have no resistance mechanism against singlet oxygen [150].

A better understanding of the molecular mechanisms underlying the formation and maintenance of the mixed species biofilm is crucial for the development of their effective treatments in the future.

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Section 4

New Therapeutic and Management

Chapter 7

Nanoparticles as New Therapeutic Agents against *Candida albicans*

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Abstract

Candida albicans is an opportunistic dimorphic yeast. This organism is pathogen associated to superficial and systemic infections. Actually, *Candida albicans* represents an emergent pathogen especially in a patient with some immunity compromises. Added to this, the use of antifungal in an indiscriminate form has increased the resistance of the existing drugs. In this aspect, the nanotechnology generates the possibility of creating new therapeutic agents. Nanoparticles are structures of 1–100 nm with special physicochemical characteristics that allow it to function as therapeutic agents or as carriers of these. Palladium, silver, and gold metallic nanoparticles and iron, titanium, zinc, and copper oxides have been used as growth inhibitors. These nanoparticles have been proved alone or in form of nanocomposites. The objective of this chapter is to describe the state of the art of the use of nanoparticles as inhibitors of the growth of *Candida albicans*, as well as the most relevant results regarding the mechanisms involved in this inhibition.

Keywords: nanotechnology, resistance, metallic and oxide nanoparticles

1. Introduction

Candidiasis is one of the most common infections worldwide. These diseases are generated by *Candida albicans* and *Candida* non-*albicans* group. *Candida albicans* is an opportunistic dimorphic yeast. It is a commensal organism in gastrointestinal, respiratory, and genitourinary

tracts. Normally, it is a harmless organism that can turn into an opportunistic organism in immunocompromised or immunologically deficient individuals [1–3].

Candida albicans represents the predominant yeast in oral, genital, and cutaneous infections (≥90%) [2, 4, 5]. However, the invasion of blood torrent by *Candida* species is the infection more frequently diagnosed. The frequency of *Candida albicans* isolated in candidemia is of 46.3%, followed by *C. glabrata* (24.4%) [3, 6]. The mortality frequency in patients with invasive candidemia ranges from 40 to >70%, generating a loss of about 1.7 billion dollars every year [1, 7].

For the treatment of invasive infection by *Candida*, few options are available. The polyenes (amphotericin B and nystatin) are the chosen treatment by excellence. However other antifungals are used as azoles [8]. The azoles (fluconazole and itraconazole) and new azoles (ravuconazole, posaconazole, and voriconazole) are selected for prophylaxis, empirical therapy, and diagnostic-driven therapy [9]. The use of echinocandins (caspofungin, anidulafungin, and micafungin) is driven to therapy on invasive candidiasis [1].

However, the adverse effects of antifungal therapy as nephrotoxicity, red blood cell (RBC) toxicity, and arrhythmias for amphotericin B, hepatotoxicity associated with fluconazole, cardiotoxicity and gastrointestinal (GIT) disturbances attributed to itraconazole, voriconazole exhibiting neurological and hepatic toxicities, and posaconazole shown to elevate serum aminotransferase levels and cause mild-to-moderate hepatic toxicity limit its use. These adverse effects are attributed to the action mechanism. Azoles and polyenes have ergosterol as a target from the cell membrane, and the ergosterol has a high similarity between ergosterol and human cholesterol [8]. Azoles inhibit the ergosterol biosynthesis, specifically the lanosterol 14α-demethylase. This generates sterol precursor accumulation, resulting in a cell membrane instability and the loss of viability [3].

The echinocandins are more specific for fungi. This therapeutic antifungal inhibits the enzyme 1,3-b-D-glucan synthase and generates the depletion of polymer synthesis and osmotic stability loss [1, 10].

Due to the discriminated antifungal agent use, *Candida albicans* has developed different resistance mechanisms. Three principal mechanisms have been reported: the efflux pump, which is a membrane transport protein that ejects the azoles out of the cell; the structural changes of the enzyme target, mutations on lanosterol 14 α-demethylase, changing the three-dimensional structure, and, as consequence, the decreased affinity for this enzyme; and the last mechanism is the increase in the production of the target enzyme; this generates the inhibition of only a portion of the enzyme, and the rest are still active [1–3, 8, 11].

On the other hand, the survival characteristics of *Candida albicans* in the host make this organism difficult to kill. The virulence factors include the biofilm formation capacity, the yeast-hyphae morphogenesis, the adhesion to surfaces, and the capacity to secrete enzymes as hydrolases.

Because of this, the research for more specific antifungal therapies has been the focus of attention. In this area, the nanotechnology offers an opportunity to design and create a new

pharmaceutical agent that can be specific. As pharmaceutical agent nanoparticles such as copper, silver, and palladium, among others, have been employed in order to inhibit the growth of any microorganism, in particular, *Candida albicans*.

The objective of this chapter is to describe the state of the art of the use of nanoparticles as inhibitors of the growth of *Candida albicans*, as well as the most relevant results regarding the mechanisms involved in this inhibition.

2. *Candida albicans* taxonomy

Fungi form one of the largest eukaryotic kingdoms; they have a very broad function of decomposing biopolymers and different compounds in dead or alive hosts, as well as synthesizing bioactive compounds. The yeast *Candida albicans* originally was described as Blastomycetes class belonging to Monilial order and Cryptococcaceae family. Actually, with gene sequencing, the new taxonomy classifies *Candida albicans* into *Ascomycota*, (dikaryon-producing fungi), *Hemiascomycetes* subclass, and *Saccharomycotina* order [12, 13].

Candida albicans is a common commensal on mucosal surfaces under normal conditions, mainly of the gastrointestinal and vaginal tracts. The commensal stage is frequently described as harmless to the host, and the fungus is controlled by the normal microbiota, epithelial barriers, and the innate immune system [14, 15]. This is the most important opportunistic medical pathogenic fungus, causing candidiasis, which is an acute, subacute, or chronic infection involving any part of the body [15]. The weakening of the defense mechanisms of the host and the adaptation capacity of the microorganism turn this fungus into an aggressive pathogen, starting with superficial infections into a systemic process, mainly in immunosuppressed or HIV patients [16].

3. *Candida albicans* cell wall

Candida albicans cell wall is composed mainly of carbohydrates; three basic main constituents are polysaccharides:

1. Branched polymers of glucose with β-1,3 and β-1,6 bonds, they are called β-glucans.
2. Unbranched polymers of N-acetyl-D-glucosamine (GlcNAc) with β-1,4 bonds; the chitin, which contributes to the fiber insolubility; and theβ-1,3 glucan-chitin complex, which is the major constituent of the inner wall. β-1,6 glucan links the components of the inner and outer walls; thus, chitin and β-1,3 are structural polysaccharides and convey strength and shape to the cell wall [15, 17, 18].
3. Finally mannose polymers, mannans, covalently associated with proteins (glucomannan proteins).

The mannan of the outer cell wall is less structured but allows this fungus breakage of epithelial barriers or dysfunction of the immune system converting *Candida albicans* from a commensal to a pathogen. The cell walls also contain proteins, 6–25%, and minor amounts of lipids, 1–7% [15, 17, 18].

This arrangement is responsible for its cell morphology and provides protection to the cell against physical, chemical, and biological (immune host system) aggression [16].

Likewise, the cell wall participates in both growth and morphological transitions between yeast and hyphae; this conversion from the unicellular yeast to multicellular filamentous fungus is essential for *Candida* virulence [19]. It has cell surface proteins linked to glycosylphosphatidylinositol (GPI) [20], including an N-terminal signal sequence and a C-terminal GPI-anchor addition signal; some of these proteins are secreted to the extracellular environment, but do not remain cell associated. Some of them are hydrolytic enzymes, which hydrolyze complex substrates into small units, to carry out into the cell as a source of nutrition. If these polymers are host targets, all of these facilitate colonization or invasion of *Candida albicans*, and then such enzymes also function as virulence factors [17]. For therapeutic strategies, to establish the functional relationships between *Candida albicans* cell wall's proteins and virulence is a challenge [20].

4. *Candida albicans* virulence factors

Candida spp. invade several anatomical hosts, mainly in patients who are immunocompromised or debilitated. This organism expresses several virulence factors that allow the host-pathogen interaction and that lead to the establishment of infection [21, 22].

Virulence factors include host recognition biomolecules (adhesins) due to cell surface hydrophobicity which enhanced adherence and resistance to phagocytosis; another important factor is its ability to grow either in yeast or in hyphal form (dimorphism, especially in the presence of N-acetyl-D-glucosamine) [23], secreting aspartyl proteases and phospholipases [21].

The phenomenon of the quorum sensing (QS) has been described as a contributor to morphogenesis, playing a role in regulating the yeast to filamentous fungus; one of the conditions is the dependence on cell density associated with the regulation by QS. The QS has an important role in the ability of *Candida albicans* to be virulent and survive within the host [23]. *Candida albicans* uses multiple redundant and/or synergistic signaling pathways to integrate host signals to promote hyphal development, tissue invasion, and virulence during infection, exacerbating the pathogenesis of invasive candidiasis [24].

5. *Candida albicans* biofilm formation

Candida virulence is a result of its capacity to form biofilms on the surface of biomaterials and on catheters and prosthetic devices [25–27]. *Candida albicans* biofilms are composed of hyphae,

pseudohyphae, yeast cells, and an extracellular matrix, where the hyphae play an integral role within this complex [26]. Biofilm formation presents an initial adherence of yeast cells (0–2 h), followed by germination and microcolony formation (2–4 h), filamentation (4–6 h), monolayer development (6–8 h), proliferation (8–24 h), and maturation (24–48 h). Serum and saliva preconditioning films and *Candida albicans* preconditioning films increased the initial attachment of the fungus but had minimal effect on subsequent biofilm formation [25, 28]. This kind of biofilm has clinical repercussions because it is the target of both chemical and immunological antifungals. Due of their intrinsic resistance to almost all antifungals in clinical use, increased resistance to this therapy, and the ability of cells within biofilms to withstand host immune defenses, the antifungal resistance of biofilms results most probably from the conjunction of several mechanisms that act in a time-dependent manner [26].

6. *Candida albicans* resistance mechanisms

In order for *Candida albicans* to survive, these microorganisms have developed diverse mechanisms. Biofilm formation, enzyme modification, and the pump efflux are the mechanisms reported to antibiotic resistance [29]. As described previously, biofilms are a complex of cells, DNA, and polymeric matrix, among others. The polymeric matrix has a crucial role in the antifungal resistance since it does not allow antibiotic penetration inside the biofilm. This phenomenon helps the internal cell to survive [25].

Resistance to azole antifungal has been described in three forms: [1] change in the affinity of cytochrome P450 sterol 14α-demethylase (gen Erg11p), [2] increase in the enzyme expression, and [3] changes in the sterol desaturase. Other alterations in an enzyme involved in the cell wall construction have been described [29, 30].

The pump efflux ejects the azoles outside the cell, without any effect inside of it. This efflux is mediated by an ATP-binding cassette (ABC) and the major facilitator superfamily. This is a complex family of genes that involve several CDR genes, including core membrane pore composed by ABC segments in the membrane; two segments are in the cytosolic side, which provide energy to pump the antibiotic outside of the cell [25, 29, 30].

It is an important highlight that this resistance could be a derivative in the resistance to other antifungals.

Candida albicans resistance could be due to one or a combination of all the resistance mechanisms described.

7. Nanotechnology as an antifungal

Nanotechnology is a new technology which manipulates the matter in a nanoscale order (1×10^{-9} m). In this order, the nanoparticles have been used as antimicrobial, as antifungal, or as carried molecules. Nanoparticles have a size from 1–100 nm on any of this dimension. In

this scale, nanoparticles have particularity physicochemical characteristics [31]. Within these characteristics, their electron configuration confers an extraordinary quantum effect. These quantum effects enhance their pharmacokinetic effect and generate nanoparticles that can be used as a protein, antibodies, or inclusive specific molecule carriers [32].

As an antifungal, nanoparticles show cell wall damage, an oxidative stress increase, and DNA interaction [33]. Nevertheless, the NPs toxicity mechanisms are dependent on the nanoparticle nature, size, and shape and capping nature, among other characteristics.

This chapter reviewed some NPs characteristics such as nanoparticle nature, synthesis, size, and shape and their influence on the toxic mechanisms in *Candida albicans* and their influence on the virulence factors.

7.1. Silver nanoparticles

Silver has been used for several decades as a disinfectant. Inclusively, silver nitrate was used in burns in order to control infections. However, with the discovery of penicillin, the use of silver was slow. Nowadays, the increase of resistance to antifungal puts attention again on the use of silver as an antibiotic, though the use of silver ions implies complications in their solubility and availability [34].

In this sense, the silver nanoparticles (AgNPs) offer a possibility. AgNPs offer the possibility of using minimal doses and act in microorganisms which present antibiotic resistance or decrease the virulence factors.

The use of different AgNPs synthesis methods caused different effects. The general method is the salt reduction by a reducing agent. However, the addition stabilizing agent has been proved. The stabilizing or capping agent limits the NPs' growth, avoids the agglomeration, and controls the shape, size, and superficial charge [35].

The NP synthesis by plant extract or secondary metabolites from microorganisms offers a new possibility. The phenolic compounds, proteins, anthocyanin, and carboxylic acid, among others, work as reducing agent and stabilizing or capping agent [35, 36].

The green AgNPs synthesis through the use of microorganisms as the use of Actinobacteria *Pilimelia columellifera* subsp. pallida [37], *Bacillus* species [38, 39], filamentous fungi *Monascus purpureus* [7], and Macromycetes as *Pleurotus sajor-caju* [40], as well as extract plant as *Tulsi* leaf extract [41] or extract from *Phoenix dactylifera* [34] has been proven. All these methods present inhibition of *Candida albicans*, with different efficiencies (**Table 1**).

The microorganisms *Bacillus amyloliquefaciens* 1853 and *Bacillus subtilis* 10,833 have been used for the AgNPs synthesis. Culture media were employed as a reducing agent. The scanning electronic microscopy has shown AgNPs produced with capping. *Bacillus* strains have been used in the AgNPS production. *Bacillus amyloliquefaciens* strain 1853 produced AgNPs with a size of 1.071 and other of 73.83 nm. The *Bacillus* subtilis strain 10,833 produced tree population sizes of AgNPs at 1.863, 10.866, and 135.78 nm. AgNPs produced by both microorganisms were employed to evaluate the *Candida albicans* inhibition. No differences were found between AgNPs; inclusive addition of antibiotic with the AgNPs does not improve the inhibition [38].

Inhibition test	NPs size (nm)	Synthesis method	Capped	Reference
18 mm (inhibition zone)	15–20	*Cassia roxburghii* + *Ketoconazole*	NR	Moteriya et al. 2017 [42]
64 μg/mL (MIC)	12.7	*Pilimelia columellifera* subsp. pallida SL19	Proteins	Wypij et al. 2017 [37]
16.7 ± 0.25 mm (inhibition zone)	2–8	*Monascus purpureus*	NR	El-Baz et al., 2016 [7]
10 μg/mL (BIC)	40–55	*Dodonaea viscosa* and *Hyptis suaveolens*	Detection of functional groups as phenol, alcohol, proteins, heterocyclic amines	Muthamil et al. 2018 [43]
40 μg/mL (MIC)	5–95	*Bacillus safensis*	Proteins	Lateef et al. 2015 [38]
12 mm (inhibition zone with 30 μL of solution)	18–46	*Gracilaria corticata*	Detection of functional groups as alcohol, carbonyl, amino groups	Kumar et al. 2013 [44]
250 μg/mL (MIC) and 500 μg/mL (MFC)	3.77–33.22	*Pleurotus sajor-caju*	NR	Musa et al. 2018 [40]
60 μg/mL (MIC_{90}) and 120 (MFC)	2–7	Tulsi leaf extract	NR	Khatoon et al., 2015 [45]
20 mm (inhibition zone with 80 μg/20 mL)	21.65–41.05	*Phoenix dactylifera*	Proteins	Oves et al. 2018 [34]
75 ppm reduce 83%/150 ppm (MFC)	70	Red cabbage	Anthocyanin compounds	Ocsoy et al. 2017 [46]
08 μg/mL (MIC)	9–130	*Enterococcus faecalis*	NR	Ashajyothi 2016 [47]
40–60 μg/mL(MIC)	4–39	Citrus lemon aqueous Juice + CTAB	Surrounded by a layer	Rahisuddin et al. 2015 [48]
10.8 ± 0.8 (inhibition zone)	12–85	Clove extract	Detection of functional groups such as methoxy, alcohol, carboxilic acid, aliphatic group	Parlinska-Wojtan et al. 2017 [49]
21–12 cm in combination with antifungal (inhibition zone)	10–90	Flower broth of *Tagetes erecta*	NR	Padalia et al. 2015 [50]
12.14 μg/mL (MIC)	47.0 ± 2.0	*J. curcas*	Protein layer	Kumar et al. 2017 [51]
10.78 μg/mL (MIC)	7.6 ± 0.5	*L. grandis*		

Inhibition test	NPs size (nm)	Synthesis method	Capped	Reference
70% fold increase (combination with antifungal, inhibition zone)	1.836–135.78	*B. subtilis* 10,833	NR	Ghiut et al. 2018 [39]
50% fold increase (combination with antifungal, inhibition zone)	1.012–73.83	*B. amyloliquefaciens*	NR	
14 mm(inhibition zone)	5–10	*Ocimum sanctum* + CTAB	Not visible	Aazam and Zaheer 2016 [35]
30 μg/mL (MIC)	4–36	Actinobacterial strain SF23	NR	Anasane et al. 2016 [52]
40 μg/mL (MIC)	8–60	Actinobacteria C9	NR	

MIC, minimal inhibition concentration.
MFC, minimal fungicide concentration.
NR, not reported.
BIC, biofilm inhibitory concentration.

Table 1. Biogenic synthesized AgNPs against ***Candida albicans.***

Clove extract was used in order to produce AgNPs. The high content of eugenol, β-caryophyllene, humulene, chavicol, methyl salicylate, α-ylangene, and eugenone; flavonoids such as eugenin, rhamnetin, kaempferol, and eugenitin; triterpenoids like oleanolic acid, stigmasterol, and campesterol; and several sesquiterpenes allowed the clove to act as a reducing and capping agent in AgNPs synthesis. The DLS analysis showed AgNPs size of 12 and 85 nm. The AgNPs produced to generate a complete inhibition of *Candida albicans* in 40-fold dilution were applied. The results obtained suggest that the functional groups' incorporation into the AgNPs generates a synergism between clove and NPs [49].

In order to evaluate the AgNPs effect on biofilms, some AgNPs have been evaluated. As described, biofilms are a *Candida albicans* virulence factor. The change from yeast to hyphae is crucial for the biofilm process. In this process, *Candida albicans* produces a polysaccharide matrix, carbohydrates, proteins, and signaling molecules [43]. Changes in the structural biofilm conformation of *Candida albicans* exposed to AgNPs were reported [28, 51, 53, 54].

The use of latex plants in the AgNPs green synthesis was proved. *J. curcas* and *L. grandis* extracts produced AgNPs with a size of 47 ± 2.0 nm and 7.6 ± 0.5 nm, respectively. The Fourier transform infrared (FTIR) spectra suggest the incorporation of phenolic OH, amides, amines, and aldehyde in the AgNPs as a stabilizing agent. The smallest AgNPs showed a better effect in plankton and biofilms of *Candida albicans* than the big ones. The AgNPs synthesized proved to be efficient in the inhibition of biofilm formation, changes in morphology and cell lysis when candida albicans was exposed [51].

Also, silver nitrate chemical reduction by sodium citrate was evaluated in intermediate and mature *Candida albicans* biofilms. The preformatted biofilms were exposed to 54 μg/mL of AgNPs. After 5 h of exposition, biofilms showed a decrease in the number of viable cells, and no differences were found in intermediate or mature biofilm [28].

The results obtained show the importance of the NPs size, being the smallest nanoparticles which show the highest inhibition [51]. Lara et al. (2015) showed that biofilm exposed to AgNPs exhibits a few *Candida albicans* filamentous formations. Electron microscopy analysis showed AgNPs accumulation and internalization [55].

Besides the AgNPs effect in biofilm, the AgNPs have been used alone showing the inhibition, cell wall damage, and incorporation of Ag into the cell. It was used in combination with different antifungals amphotericin b [51, 56] or nystatin and chlorhexidine digluconate, showing a synergic effect [54]. Other molecules as cationic carboxilane have been used, proving a high *Candida albicans* inhibition with small AgNPs concentration [1.8 mg/mL; [57]]. Also, the AgNPs have been capped with L-3,4-dihydroxyphenyl-alanine, showing a minimal fungicide concentration of 31.2–62.5 μg/mL [58].

The direction in the investigation with silver nanoparticles has been driven in the green synthesis due to the low toxicity generated in the human cell and the increase of the toxicity in bacteria and yeast [59]. Their chemical characteristics as a metallic oxidative state and superficial area, among others, allow the NPs to interact with the microorganism and inhibit their growth.

7.2. Zinc oxide nanoparticles

Due to ZnO low toxicity, ZnO has been used in a medical device such as drug carriers, antibacterial agents, and bioimaging probes, among others. For this reason, the ZnO nanoparticles (ZnONPs) have been used in *Candida albicans* control. The ZnONPs application is reflected by the annual production between 550 and 5500. This is more than 10–100 times higher than any other nanoparticle [60]. As the AgNPs, the ZnONPs characteristics depend on the size, synthesis method, and superficial charge, among others [61]. In order to control these characteristics, the green synthesis method has been used to fabricate ZnONPs with a size, shape, and superficial charge with the desirable characteristic [62, 63].

A promissory ZnONPs application area is their use as antimicrobial. Their effect has been evaluated in soil, plants, bacteria, and fungi [60, 64–66] ZnONPs different synthesis methods in ZnONPs have a big influence in the *Candida albicans* inhibition [68].

The use of *Atalantia monophylla* extracts produced ZnONPs 30 nm that have effect inhibition in *Candida albicans* growth. The author proposed the ZnONPs interaction with the cell wall, penetration to the cell, ion liberation, and the oxidative stress production, triggering the protein, lipid, and DNA oxidation [67].

The microwave aqueous extract from *Suaeda aegyptiaca* produced ZnONPs with a size of 80 nm with a capping produced by metabolites from the plant. The effect of the capping was studied. ZnONPs produced by precipitation method were studied and compared with the plant extract produced. ZnONPs *Suaeda aegyptiaca* produced a major inhibitory effect than the ZnONPs without capping agent, suggesting a synergic effect [68].

Extracts for different plants have been proved to synthesize ZnONPs in order to evaluate the toxicity of the resulting nanoparticles in *Candida albicans* inhibition (**Table 2**).

ZnONPs hybrid has been synthesized using chitosan, gelatin, and polystyrene, among others [75–77].

Dhillon et al. 2014 [75] used chitosan as a capping and bridge to carry on citric acid, glycerol, starch, and whey powder using the nanospray drying method. The ZnONPs produced have a size range of 93.2–402.5 nm. The smallest ZnONPs were the chitosan starch NPs. Aggregation effect was observed in the largest ZnONPs; this phenomenon could be explained by the superficial charge. However, these ZnONPs have a small effect in *Candida albicans* inhibition.

Collagen was used as a reducing and capping agent. The ZnONPs produced with this method showed a size between 20 and 50 nm negatively charged. ZnONPs coated by collagen

Inhibition test	NPs size (nm)	Synthesis method	Capped	Reference
24 mm (inhibition zone)	30	*Atalantia monophylla*	NR	Vijayakumar et al. 2018 [67]
80 μM (MIC)/>2590 μM (MFC)	8	*Chelidonium majus*	NR	Dobrucka et al. 2018 [69]
34 ± 1.28 mm (inhibition zone with 100 g/ml)	32–40	*Glycosmis pentaphylla*	Alkane, C=O stretching, other groups	Vijayakumar et al., 2018 [70]
25 μg/mL (MIC)/50 μg/mL (MFC)	18	*Azadirachta indica*	Proteins	Elumalai and Velmurugan 2015 [71]
Strong activity	36	*S. myriocystum*	Protein, carbohydrates, flavonoids, tannins, mannitol	Nagarajan and Kuppusamy 2013 [72]
12.5 mm (inhibition zone with 150 mg/ml)	68.64	*Cassia auriculata*	Alkene, alcohol, ether, and alkane	Padalia et al. 2018 [73]
20.3 ± 0.57 mm (inhibition zone with 200 μg/mL)/25 μg/mL (MIC) 50 μg/mL (MFC)	10–30	*Vitex trifolia*	Proteins, alkanes, aromatic group	Elumalai et al. 2015 [71]
18.01 mm (inhibition zone 100 μg/mL)	80	*Suaeda aegyptiaca*	Alcohol, aldehyde, amine	Rajabi et al. 2018 [68]
Inhibition test	**NPs size (nm)**	**Synthesis method**	**Capped**	**Reference**
24 mm (zone inhibition)	30 nm	*Atalantia monophylla*	NR	Vijayakumar et al. 2018 [67]
80 μM (MIC)/>2590 μM (MFC)	8 nm	*Chelidonium majus*	NR	Dobrucka et al. 2018 [74]

MIC, minimal inhibition concentration.
MFC, minimal fungicide concentration.
NR, not reported.

Table 2. Biogenic synthesized ZnONPs against *Candida albicans.*

showed a MIC of 99.7 ± 0.99 μg/mL, whereas the control with zinc acetate showed an MIC of 297.9 ± 2.0 μg/mL. Biofilms exposed to increase the concentration of ZnONPs showed inhibition as a result of the ZnONPs concentration [78].

Gelatin was used for the ZnONPs as well. ZnONPs produced with gelatin showed a diameter of 20 nm with a negative superficial charge. The ZnONPs produced were employed to biofilm exposition. ZnONPs in a concentration of 50 μg/mL showed a thickness diminution, resulting in a weak adherence biofilm compared with the biofilm treated with fluconazole [72].

Also, egg albumin was used as a ZnONPs template. The synthesis method produced spherical ZnONPs with a range between 10 and 20 nm. The FITR analysis showed the interaction of egg albumin and ZnONPs. In this work, ZnONPs characterization in the culture media showed importance. The use of 45 μg/ml in Sabouraud's dextrose (SD) nutrient media does not significantly affect ZnONPs stability, size, and integrity. The synthesized ZnONPs showed *Candida albicans* inhibition in a dose-dependent manner. Changes in the *Candida albicans* morphology show cavity formation in cells exposed to 15 μg/ml [79].

ZnONPs were evaluated both alone and as a part of nanocomposites with polystyrene. The use of ZnONPs alone showed *Candida albicans* inhibition. However, when ZnONPs are used in nanocomposites, a major concentration of ZnONPs to inhibit *Candida albicans* is necessary [77].

Nanocomposites with ZnONPs have been produced with different materials in order to increase the growth inhibition of microorganisms [45, 46]. However, the results of the antifungal activity were contradictory; ZnONPs capped by surfactant in cotton fiber have proved the inhibition of *Candida albicans* in 94% [68], while the incorporation of ZnONPs in polylactic acid films does not inhibit the *Candida albicans* growth [80].

The principal ZnONPs toxicity mechanisms reported are (1) ion liberation, (2) interaction of ZnONPs with the cell wall, and (3) stress oxidative generation [81]. The ion liberation and the interaction of NPs with the cell trigger the generation of oxidative stress; with that the cell lost their viability [79].

7.3. Copper oxide nanoparticles

Copper exhibits good characteristics such as its antimicrobial activity, chemical stability, and thermal resistance. Due to their toxicity characteristic, copper has been used by Egyptians for water disinfection. The Aztecs used copper to treat sore throats, and the Persian and Indians used it to treat eye infection and venereal ulcers [82].

Copper has an advantage in comparison with other materials, is cheaper than any other, and is easy to oxidize to copper oxide nanoparticles (CuONPs). The CuONPs can easily make nanocomposites with polymers, macromolecules inclusive of other metals [83, 84].

The CuONPs have recovered importance in the investigation due to their low cost and the variety of applications. CuONPs have been used in the pharmaceutical, medicine, and electronic industries, among others.

Diverse synthesis methods have been proved to create CuONPs including chemical [85], electrochemical [86], and green synthesis [87].

The synthesis method influences the characteristics as the size, shape, and agglomeration of the CuONPs [88].

CuONP electrochemical production with different solvents and reaction times produces different NPs sizes from 3 to 200 nm. The CuONPs were obtained in water-acetonitrile, with sodium hydroxide as the electrolyte. The CuONPs obtained in these conditions showed a size around 20 nm. The *Candida albicans* exposition to 25 and 50 μg/ml generates a delay in the growth curves. Added to that, 50 μg/ml inhibited completely *Candida albicans* growth [86].

CuONPs prepared by precipitation method coated with acetate were evaluated together with fluconazole. CuONPs coated with acetate were more toxic than CuONPs without coating. CuONPs in combination with fluconazole showed the complete *Candida albicans* inhibition. The fractional inhibitory concentration index (FICI) suggested the additive to moderate synergism between CuONPs and fluconazole [89].

The use of dispersant as ethylene glycol and Tween 80 proved to be efficient in the production of CuONPs capable to the *Candida albicans* growth inhibition. Inhibition with CuONPs was more efficient than the exposition to itraconazole [90]. Also, the effect of CuONPs was evaluated in *Candida albicans* from oral cavities [91], showing that strain isolated from the patients needs until 1000 ppm to generate an inhibitory effect in *Candida albicans*.

CuONPs capped with pyrimidine derivatives have shown a size of 10 nm. The CuONPs showed interaction with DNA and antioxidant activity. However, the CuONPs showed *Candida albicans* inhibition [92].

Nanocomposites with the incorporation of Fe [93] Cd and Ba CuONPs [94], as well as the incorporation of CuONPs into cotton fiber [95, 96], polyurethane [97], polyester [98], copolymer microgel [99], and open polyurethane foams with starch powder [100], have been proved be efficient in the *Candida albicans* growth inhibition.

As in the other nanoparticles, the green synthesis has captured the investigators' attention. Its methodology does not produce toxic subproduct in the nanoparticle production, and it covers the nanoparticles avoiding the agglomeration [31, 84, 87].

Extracts of garlic and ginger have been used for the oxidation of copper. Garlic produces the smallest CuONPs, with a size around 14.62–22.80 nm. However, ginger shows a big quantity of very small nanoparticles. *Candida albicans* has shown major inhibition when exposed to ginger ethanol extract NPs [101]. Few reports about the use of biological extract for the synthesis of CuONPs that inhibited *Candida albicans* were founded (**Table 3**).

Cu^{2+} ions realized from CuONPs are the principal toxicity mechanisms reported. However, it is not the only one. Some authors reported the CuONP accumulation in the cell wall as well as the oxidative stress increase. However, more studies are necessary in order to understand the NPs toxicity mechanisms.

Inhibition test	NPs size (nm)	Synthesis method	Capped	Reference
15.0 c ± 0.57735 (inhibition zone with 62 μg/mL)/1.93 ± 0.76376 (MIC)	31.7	Alginate	NR	El-Batal et al. 2018 [102]
10.5 (zone inhibition)	26 ± 4	*Acalypha indica*	NR	Sivaraj et al. 2014 [103]
22.5 mm (inhibition zone)	Nanostructures ~8000	*Viburnum opulus*	$Cu_3(PO4)_2$ $3H_2O$	Ildiz et al. 2017 [104]
20 mm (inhibition zone with 50 μg/mL)	NR	*Sargassum polycystum*	NR	Vishnu et al. 2016 [105]
0.15 Log_{10} growth reduction	22 ± 1 nm	Cellulose, chitosan, and keratin nanocomposites	Cellulose, chitosan, and keratin	Tram et al., 2017 [106]

Table 3. Biogenic synthesized CuONPs against *Candida albicans*.

7.4. Other nanoparticles

Other metal nanoparticles as gold [AuNPs, [36]], palladium [PdNPs, [91]], and selenium [SeNPs, [92]], among others, have been proven. The microorganisms' elimination by all these NPs has been proved to be effective.

Chemical reduction of palladium salt has produced PdNPs in an average size of 9 nm (±3.9 nm). The PdNPs showed a significative *Candida albicans* growth reduction at 150 ppm [107].

SeNPs were prepared by the reduction of selenium chloride inside *K. pneumoniae*. This synthesis produced SeNPs with a size range from 90 to 320 nm. The concentration necessary for growth inhibition of *Candida albicans* was 2000 μg/mL [109]. Another strategy for the *Candida albicans* inhibition induced the SeNPs formation inside of *Lactobacillus* and then used them as an antifungal. This strategy worked with *L. plantarum* and *L. johnsonii. Candida albicans* exposed to both microorganisms becomes more efficient than bacterium without NPs [108].

Schiff base ligand 2-((4,6-dihethoxypyr-imidine-2-yl)methyleneenamino)-6-methoxyphenol has been used in order to capped AuNPs and platinum nanoparticles (PtNPs). Nanoparticles synthetized presented a layer. The NPs showed a granular and spherical shape with size of 38.14 ± 4.5 and 58.64 ± 3.0 nm, respectively. Both nanoparticles showed greater inhibition than amphotericin [110].

Biogenic PdNP production with watery extract of *Moringa oleifera* flower was evaluated for the *Candida albicans* inhibition. However, these PdNPs had no effect in *Candida albicans* [111].

Candida albicans exposition to tellurium NPs produces complete inhibition with of ≥2000 μg/mL. Besides, the results showed a squalene accumulation and increase in the expression levels of the ERG1 gene of squalene monooxygenase enzyme [112].

7.5. Nanoparticle characteristics and toxicity and *Candida albicans* inhibition growth

The most important NPs characteristics are the size, shape, composition, superficial charge, and hydrodynamic. These characteristics directly influence their capacity to interact with the molecules or cells. Ocsoy et al. (2017) [46] indicate that the size is strictly related to charge density. The smallest of the NPs has the biggest charge density. This explains the big attraction between small NPs and their agglomeration. The NPs agglomeration is a phenomenon that changes their capacity to work as a caring or their toxicokinetic characteristic [33, 107]. In order to improve the distribution and decrease the agglomeration, a surfactant as Pluronic F® has been added to the synthesis process (**Figure 1**). Other surfactants as CTAB, SDS, cation surfactants [113], or PVP have been proved to be efficient [114].

Another synthesis methodology that improves these characteristics is the use of subproducts from plants, algae, bacteria, fungi, yeast, etc. [115]. The high content of proteins, reducing sugars or anthocyanins, works as reducing agent; additionally, some of these molecules are adhered in the NPs surface [31]. This addition gives different characteristics to the NPs produced by chemical reduction. The NPs obtained with biogenic methods offer homogeneous NPs with antioxidant characteristics that allow the addition of other molecules and control their toxicity characteristics.

The biogenic NPs production has proven to be efficient inhibiting the *Candida albicans* growth (**Tables 1–3**). The differences in the concentration necessary to inhibit their growth are directly related to NPs size, the capping nature, and the microorganisms evaluated [116]. These are evident in the work of Anasane et al. 2016 [52] et al. (2013), where two microorganisms were evaluated in the production of AgNPs, obtaining two NPs sizes. The AgNPs production by the strain SF23 was smaller than that produced by the strain C9. *Candida albicans* presented an MIC of 30 μg/mL with the small AgNPs (size 4–36 nm). Wypij et al. (2017) [37] reported the AgNPs synthesis with *Pilimelia columellifera* subsp. *pallida* SL19, with a capping

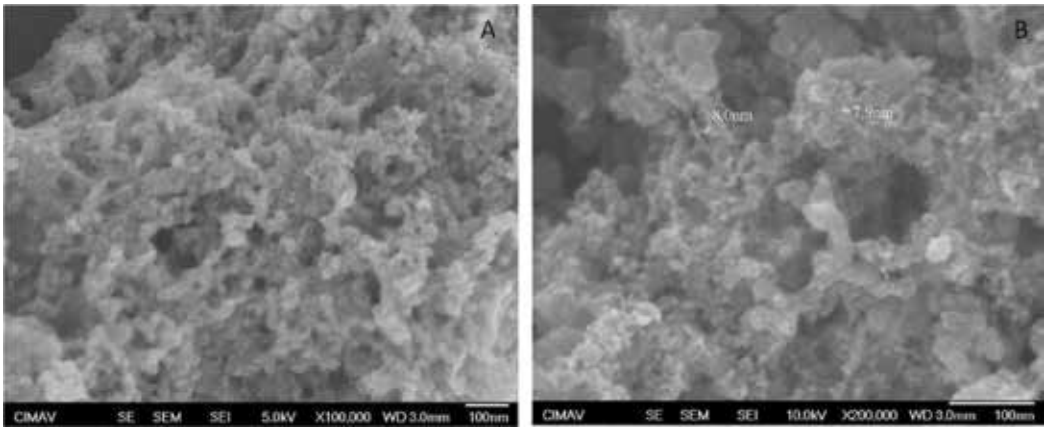

Figure 1. Scanning electronic microscopy of PdNPs produced by chemical reduction. (A) Without surfactant. (B) With Pluronic F® (5% W/V).

of proteins. The AgNPs size was on average 12.7 nm with a growth inhibition of 64 μg/mL. Tulsi leaf extract has been used to produce AgNPs with a size of 2–7 nm. The fungicide concentration in the AgNPs produced was of 120 μg/mL [25]. The production of ZnONPs by *Azadirachta indica* [61], *Vitex trifolia* [64], and *Suaeda aegyptiaca* [65], among others (**Table 2**), has been evaluated. The extract produced different ZnONPs sizes and capping (**Table 2**) that influence the growth inhibition.

Due to the NPs size (nanometer scale), diverse toxicity mechanisms have been proposed (**Figure 2**). One is the direct interaction of the nanoparticle with the cell membrane. The interaction of NPs microorganism is allowed by the NPs superficial charge but also the microorganism superficial charge. This interaction generates disruption of the cell wall and the leakage of ions and the intracellular material with the microorganism's death [117–119].

Consequent to NPs dilution, the ion interaction with the cell is another toxicity mechanism. The ions are incorporated into the cell, and they could interact with thiol groups of proteins and enzymes leading to the inhibition of crucial biological activities [82]. Also, the NPs internalization has been reported; this generates the interaction of NPs and ions with molecules of biological importance as DNA or enzymes [33, 120]. Inside the cell, the NPs or the ions provoke Fenton's type reaction. Due to this, the oxidative stress increases, and the proteins, lipids, and DNA release (**Figure 2**).

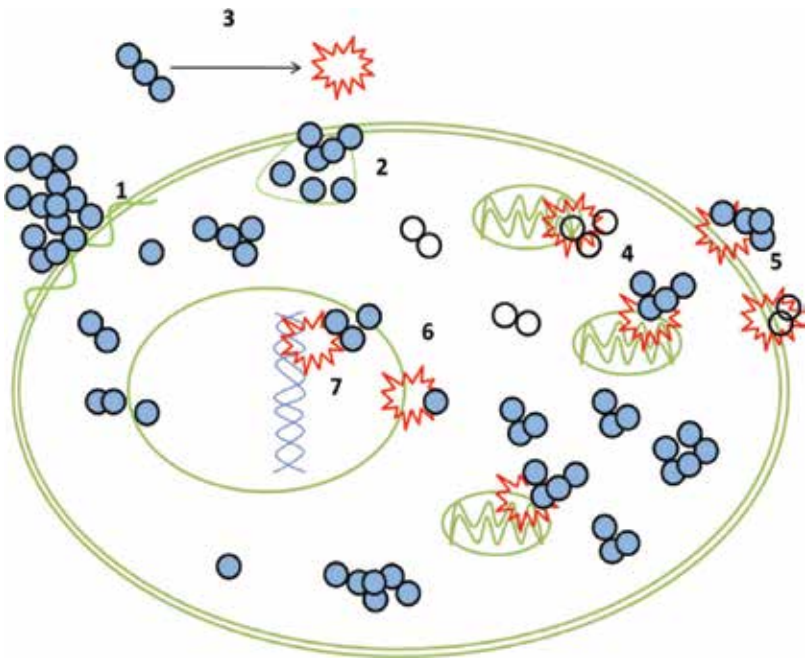

Figure 2. NPs toxicity mechanisms induced in *Candida albicans*. (1) NPs adhesion in the cell membrane causing disruption in the membrane and death. (2) NPs introduction to the cell with their posterior degradation and ion release. (3) NPs ROS generation outside the cell. (4) NPs and ions ROS generation in interaction with mitochondria. (6) Membrane lipid peroxidation with nucleus damage. (7) DNA damage by NPS and ions ROS generation.

Khan et al. (2014) [121] write "when $CoFe_2O_4P$ NCs gained a higher energy than Eg, the electrons (e^-) of $CoFe_2O_4P$ NCs were promoted across the band gap to the conduction band (Ec), which creates a hole (h+) in the valence band (Ec). These e^- in the E_C and holes in the Ev possibly have high reducing and oxidizing powers, respectively." These electron movements generate the NPs reactivity and the oxidative stress generation. The same phenomenon is reported by Setyawati et al. (2014) [122] for TiONPs. These NPs adsorbed UV light, and the adsorbed UV light generates electron excitation, creating a hole in the valence band. The electrons and holes migrate to NPs superficies where they can react with oxygen or water generating oxidative stress. The superficial e^- generates the liberation of hydroxyl increasing the mitochondria membrane depolarization and liberation of cytochrome c. Added to this, the results show DNA damage [120]. However, the oxidative stress is not the only mechanisms involved in the AgNPs toxicity; also the membrane fluidity, ergosterol content, and cellular and ultrastructure morphology are altered [123]. The squalene monooxygenase expression in *Candida albicans* exposed to TeNPs showed an increase [112]. The squalene monooxygenase catalyzes the conversion of squalene to 2,3-oxidosqualene; inhibition in this step generates the squalene increase and the disruption of the *Candida albicans* cell wall [107].

8. Conclusion

The antibiotic resistance increase in many microorganisms has encouraged researchers to focus their efforts on the synthesis and design of multiple effective compounds in order to combat the resistance mechanism of microorganisms. NPs offer a new and effective "antibiotic" that could work against the resistance mechanism in *Candida albicans*.

The biogenic NPs offer a new generation of NPs that work as a carrier. These NPs offer the possibility to drive the toxicity effect to a specific target, in such way the collateral damage could be diminished.

However, the NPs toxicity mechanisms are not completely understood. The oxidative stress is one of the described mechanisms; it is possible to investigate other mechanisms involving similarity to oxidative stress. More studies are necessary to understand the influence that nanoparticles have in the *Candida albicans* resistance mechanisms and if this exposition could not generate new ones.

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Conflict of interest

The authors declare no conflict of interest.

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This book highlights modern techniques of research into *Candida albicans*, especially in terms of emerging and emerged pathogenic *Candida* species. It also looks at metabolic adaptation, resistance related to environmental stress and variety of nutrients, best performing plants that inhibit *Candida*'s activities, interaction with other microbes, antifungal immunity mechanisms, and the posttherapeutic management of fungal infections. The book is a collection of very high impact research that includes a combination of biochemical, molecular biological, and medical microbiological innovative scientific techniques. It contains fascinating information that will help readers to explore and understand why *C. albicans* is different from other microbes. The authors describe this significant discovery using both bioinformatic and laboratory techniques and this uniqueness is the reason why *C. albicans* is a successful pathogenic yeast.

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