

Original Article

Transcriptional analysis of oxidative and nitrosative stress on oral opportunistic *Candida albicans*

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Abstract



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The yeast *Candida albicans* is one of the most aggressive opportunistic pathogens in immunocompromised patients. The ability of the yeast to withstand stresses and radicals is of great concern. In the present study, four isolates of *C. albicans* were taken from patients with oral candidiasis and grown on RPMI for 24 hours at 37°C. Then, they were exposed to various concentrations of oxidative (H₂O₂) and nitrosative (HNO₃) stress for two hours, and gene expression rates were measured through RT-PCR. After initial biofilm formation steps and growth validation, RNA extracted from the yeast and gene expression status were evaluated. Upon treatment with H₂O₂, the gene expression profile for ALS1, MLH1, and EXO1 showed approximately a fold increase in expression. While within HNO₃ the yeast gene expression exhibited a dramatic increase in ALS1 up to 217 folds, while others such as MLH1, HWP1, and ERG11 showed a one-fold increase in the expression rate. The findings of this research indicate a considerable expression activity within the biofilm of *Candida albicans*, increased rate of DNA mismatch repair and break fixation may indicate the ability of the yeast to tolerate high concentrations of free radicals. It paves the way toward understanding the pathogenicity of the yeast and its survival capability inside macrophages. The study also revealed that the biofilm strategy of the yeast is more active within these stresses.

Keywords: *Candida albicans*, Oxidative and nitrosative stress, Virulence biofilms, RT-PCR.

1. Introduction

Candida albicans, a yeast commonly found in the normal microbial community of the human body, can transform into a dangerous opportunistic pathogen, particularly in individuals with weakened immune systems [1]. Various risk factors, including immunosuppressive chemotherapy, prolonged catheter use, and extended broad-spectrum antibiotic treatment, have contributed to a significant rise in *Candida* infections [2, 3]. A significant obstacle to *C. albicans* treatment is their capacity to create biofilms within the human body. These biofilms serve as protective shields for the yeast, preventing antimicrobial agents and the host's immune system components from reaching it. This is achieved by forming an extracellular matrix [4].

Phagocytes mediate their innate immunological response by releasing products that damage invading microorganisms. These products include proteins such as lysozyme, peroxidases, and elastase as well as reactive oxygen species such as superoxide, hydrogen peroxide, hypochlorous acid, and hydroxyl radical and reactive nitrogen species such as nitric oxide [5]. Although it is clear that many phagocytic secretory products have direct cytotoxic potential, understanding is limited of how multiple

products interact to generate and modulate the cytotoxic response. One factor contributing to the virulence of *C. albicans* is its ability to withstand various host defense mechanisms, including oxidative stress and nitrosative stress [6]. Oxidative stress arises from the buildup of reactive oxygen species (ROS) like hydrogen peroxide, superoxide anion, and hydroxyl radical. On the other hand, nitrosative stress occurs when reactive nitrogen species (RNS) such as nitric oxide and hydrogen peroxide are produced [5].

In recent years, several studies have focused on investigating how *C. albicans* responds to oxidative stress and nitrosative stress, aiming to understand the mechanisms behind its resistance to these stressors [7]. These studies have emphasized the significance of antioxidant defense systems like the glutathione system and catalase in protecting *C. albicans* against oxidative stress. Additionally, they have highlighted the role of enzymes such as flavohaemoglobin in detoxifying nitric oxide under conditions of nitrosative stress. In the microbial world, free radicals are believed to contribute to gene mutations by creating DNA adducts resulting in mismatch pairing and DNA breakage. In addition, once passing the cell membrane of the microbes, free radicals can cause lipid damage and pro-

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tein destruction. This is through the ability of free radicals to oxidize thiols, urate, and ascorbate. It chlorinates the tyrosine bases of even the intracellular organelles, which causes protein fragmentation [8, 9]. Yeast has a mechanism to effectively deal with mutations in its DNA, which is controlled by a specific group of DNA mismatch repair genes, particularly MLH1, MSH2, and EXO1. Among these genes, EXO1 plays a crucial role in quality control, acting as a guardian against excessive mutation errors [10].

Furthermore, these mismatch repair genes have demonstrated their importance in preserving genome stability and acquiring resistance to drugs in *C. albicans*. Recent research has revealed an increased vulnerability to specific antifungal medications due to the removal of certain double-strand DNA repair genes [11]. The colonization of the oral cavity and vagina by *C. albicans*, along with its ability to cause bloodstream infections in immunocompromised individuals, can lead to the production of acetaldehyde through the breakdown of ethanol via anaerobic fermentation [9, 12–14]. Moreover, there is a known connection between the expression levels of ADH and the azole resistance genes CDR1 [14]. The metabolic activity of ALD5 has been observed to have an inverse relationship with the expression of drug-resistant genes, specifically ERG11 and CDR1, in resistant strains of *C. albicans* [15]. However, it has been demonstrated that resistance to antifungal agents arises due to increased ERG11 expression [16]. Despite these advancements, the exact mechanisms by which *C. albicans* respond to oxidative stress and nitrosative stress are still unclear. Additionally, the interaction between these two stressors and their impact on the pathogenicity of *C. albicans* has yet to be fully explored. In this study, our goal is to investigate how *C. albicans* responds to oxidative stress and nitrosative stress, and to determine the potential interplay between these two stressors in influencing the virulence of this pathogen.

2. Materials and Methods

The study's experimental and laboratory work was conducted at the Microbiology and Molecular laboratories of the Research Center of Salahaddin University and Zheen International Hospital.

2.1. *Candida* Isolates

The study involved clinical isolates of *C. albicans*. These isolates were previously collected and confirmed from oral cancer patients at Nanakali Hospital in Erbil City. During the identification process, multiple colonies were tested at each step. The colonies were subcultured, purified and further cultivated on CHROMagar™ *Candida* (CHROMagar, Kanto Chemical Co., France) to presumptively identify *C. albicans* based on their green colonies. The isolates' ability to form biofilms was tested and categorized into three grades based on visual assessment. This assessment considered the thickness, transparency, adherence, and ability to withstand transfer of the biofilm. The chosen isolates were then tested with basic minimal inhibitory concentration (MIC) testing using E-test strips (AB Biodisk, Sweden) containing Fluconazole, Itraconazole, and Voriconazole (0.002 to 256 mg/mL) [17].

2.2. Gene expression levels in response to oxidative and nitrosative stress

The impact of oxidative and nitrosative stress was investigated via selected gene expression levels. Using specific primers, as mentioned in Table 1, the gene expression for the exon DNA was conducted through RNA extraction.

The isolates were tested using a range of oxidative and nitrosative stresses. Concentration gradients were prepared to cover the clinical relevance and effects on the isolates, starting at 2mM, 4mM, and 6mM, respectively. Biofilms of *C. albicans* were grown using a modified version of the procedure outlined by [17] as described below: Day 1: *C. albicans* isolates were streaked onto Sabouraud agar (SAB) plates from -80°C freezer stocks. The plates were incubated for 2 full days at 37°C. Day 3: A solitary colony from a SAB plate was placed in 20 mL of Yeast Peptone Dextrose broth (YPD) in 40 mL tubes. The cultures were incubated in a shaking incubator at 37°C for a maximum of 16 hrs., allowing them to grow overnight. Day 4: The YPD medium was removed by centrifuging the tubes at 3000 ×g for 5 min at 4°C. The cells were washed twice in 20 ml PBS, pH 7.4, and then finally resuspended in 20 mL of PBS. The cells were counted using a hemocytometer, and a dilution of 1:10 was used to make a cell suspension with a final concentration of 1.0×10⁶ CFU/mL in an appro-

Table 1. List of primers used in the present study.

Primer's name	Sequence '5-----3',	References
ALS1-F	GACTAGTGAACCAACAAATACCAG	This study
ALS1-R	ACCAGAAGAAACAGCAGGTG	
HWP1-F	ATGACTCCAGCTGGTTC	
HWP1-R	TAGATCAAGAATGCAGC	
MLH1-F	CGTCGAGATTACGGGGATTA	[18]
MLH1-R	CCTTAATCGGCATCTGAGGA	
EXO1-F	CCGATTGGGACTCCAGTTTA	
EXO1-R	CTTGCGGTTCTCTTCTTCAT	
ERG11-F	AGCTTCATATGGTCAACAACCT	[19]
ERG11-R	CTTCTTCAGCAGAAACATCAG	
CDR1-F	GCCCTTTATCGTCCTTCAGC	[19]
CDR1-R	AGGAGTCATGGCACCAGAAA	
RIP-F	TGTCACGGTTCCCATTATGATATTT	[18]
RIP-R	TGGAATTTCCAAGTTCAATGGA	

appropriate volume of RPMI-1640 (containing L-glutamate but not sodium bicarbonate, buffered with 165 mM MOPS to a final pH of 7.4). One mL of the cell suspension was added to each well in the 24-well plate(s) (Costar) into which Thermanox cover slips (Thermanox) had been placed previously (1 plate \times 24 wells = 24 cover slips needed). The plates were sealed with parafilm and incubated for 24 hours at 37°C.

2.3. Biofilm viability using Roche XTT Procedure

To evaluate the biofilm activity and viability, the XTT reagent was activated using an activator in a ratio of 0.1 mL for every 5 mL of XTT reagent. Next, the activated reagent (200 μ L) was added to each biofilm well in the plate along with 3 blank wells for control. The plate was then covered with aluminum foil and incubated at 37°C for 2 hrs. After incubation, 100 μ L of the XTT reagent from both biofilms and controls was transferred to a new 96-well plate and OD was measured at 490 nm and 620 nm using a BMG polarimeter equipped with Polar Star Omega software. The biofilms were then harvested along with any remaining liquids into Eppendorf tubes and snap-frozen in liquid nitrogen. These biofilms were stored at -80°C for RNA extraction later.

2.4. RNA Extraction

To extract RNA, biofilms of *C. albicans* isolates from each treatment were defrosted on ice. RNA lysis buffer R (0.45 mL) was added and left to cool for 2 minutes. The tubes were gently vortexed, and the supernatants were transferred into sterile 2mm screw-capped tubes containing sterile glass beads. Then, the RNA samples were extracted using the extraction kit (Canvax, German) based on the provided procedure by the manufacture. The steps of the total RNA extraction are indicated in the below points. To begin, transfer the fungal material into a 2 mL tube. Next, add 600 μ L of RNA Buffer Lysis and vigorously mix for 60 seconds. Then, centrifuge the tube at 15,000 \times g for 2 minutes. Transfer the liquid portion into a microcentrifuge tube with a capacity of 1.5-2 mL. Then, add 600 μ L of 70% ethanol to the transferred liquid. Thoroughly mix the solution by either pipetting or vortexing it. Carefully transfer up to 700 μ L of the mixture you obtained into an RNA-prep spin column that has been placed inside a collection tube. Centrifuge the column for 15 seconds at 15,000 \times g. Afterward, dispose of the flow-through and keep the column along with the collection tube for reuse. Transfer the remaining mixture into the RNAprep spin column that you used earlier. Centrifuge the column at 15,000 \times g for 15 seconds. After centrifugation, discard the flow-through and carefully transfer the RNAprep spin column into a new collection tube. Add 700 μ L of RNA Wash Buffer-1 to the sample and centrifuge it for 15 seconds at 15,000 \times g. After centrifugation, discard the flow-through and you can reuse the collection tube. Add 500 μ L of RNA Wash Buffer-2 to the sample and centrifuge it for 15 seconds at 15,000 \times g. After centrifugation, discard the flow-through and you can reuse the collection tube. Centrifuge the sample for 90 seconds at 15,000 \times g. Then, dispose of both the collection tube and flow-through. Finally, transfer the spin column to a sterile 1.5 ml RNase-free microcentrifuge tube with care. The presence of alcohol in wash buffer-2 can potentially affect enzymatic reactions and reduce the efficiency of elution. Therefore, it is crucial to thoroughly remove

all traces of alcohol from the spin column prior to elution. Add 50-100 μ L of RNA Elution buffer onto the center of the spin column membrane. Then, centrifuge the column at 15,000 \times g for 2 minutes. Remove the spin column from the tube and transfer the tube containing the eluted RNA to a freezing rack. The isolated RNA can then be stored at -80°C for future use.

2.5. Real time PCR test

The One-Step Probe RT-PCR Kit (Canvax, German) condition started with reverse transcription for cDNA synthesis at 50°C for 10 minutes then initial denaturation at 95 °C for 3 minutes followed by 45 cycles of denaturation at 95°C for 10 s, and annealing/extension at 60°C for 45 s. To compare levels of mRNA expression between treated and healthy controls, delta-CT values were calculated.

2.6. Statistical Analysis

The data were analyzed using GraphPad Prism version 9.01 (GraphPad Inc., San Diego, CA, USA) and presented as Means \pm Standard Error of the mean (SEM). Group differences were assessed using the two-tailed Mann-Whitney test and two-tailed paired t-test to compare means within groups. For multiple datasets, the Kruskal-Wallis's test and one-way ANOVA with Geisser-Greenhouse's epsilon corrections were employed. The correlation between groups and treatments was determined with a 95% confidence interval and *p*-value.

3. Results

3.1. Isolate characteristics

The identification of the clinical *C. albicans* isolates and control *C. albicans* isolate used in this study was confirmed through conventional, cultural, biochemical and morphological approaches. The ability of the isolates to form biofilms was also analyzed by visual inspection, and oral isolates produced mature biofilms within 24 hrs., of incubation (Figure 1).

3.2. Determining the Minimum Inhibitory Concentration (MIC) for Fluconazole, voriconazole and itraconazole

The isolates were subjected to the E-test in order to determine their minimum inhibitory concentrations (MICs). Results indicate the resistance and sensitivity of the isolates that were previously tested using the National Committee for Clinical Laboratory Standards Document M (2002).

3.3. Measuring Metabolic Activity using XTT Assay

The metabolic activity of *C. albicans* biofilms was

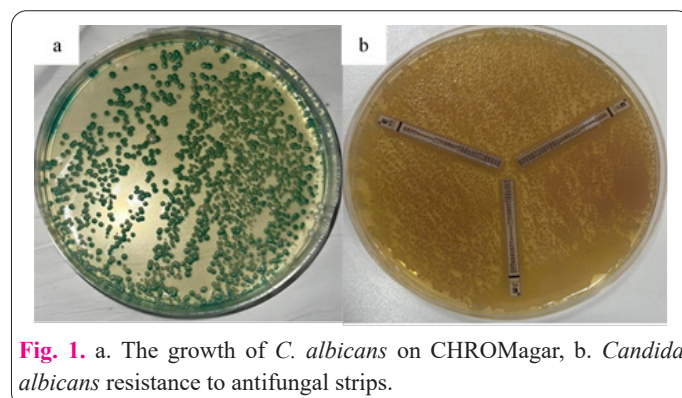


Fig. 1. a. The growth of *C. albicans* on CHROMagar, b. *Candida albicans* resistance to antifungal strips.

assessed using the XTT reduction assay, which involves the use of 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide. This method was described [18] and is depicted in Figure 2.

3.4. Measuring Metabolic Activity of Biofilms and Gene Expression

To assess the metabolic activities of biofilms, we checked the optical density (OD) at 24 hrs., (pre-treatment, 2mM, 4mM and 6mM) incubation then added the XTT solution of additional incubation at concentrations of 0 mM, 2 mM, 4 mM, and 6 mM (Figure 3).

3.5. Gene expression analysis

The expression levels of genes were analyzed using GraphPad Prism software, we compared the expression levels of genes ALS1, HWP, MLH, EXO1, ERG11, and CDR1 with that under the influence of H_2O_2 and HNO_3 stresses at four different concentrations. The results demonstrated that among the four different concentrations of H_2O_2 , the ALS1 gene exhibited the highest level of expression. However, under the influence of HNO_3 , the MLH1 gene showed the highest expression (Table 2 and Figure 4).

4. Discussion

The yeast *Candida albicans* is a common opportunistic pathogen that can cause a variety of diseases ranging from

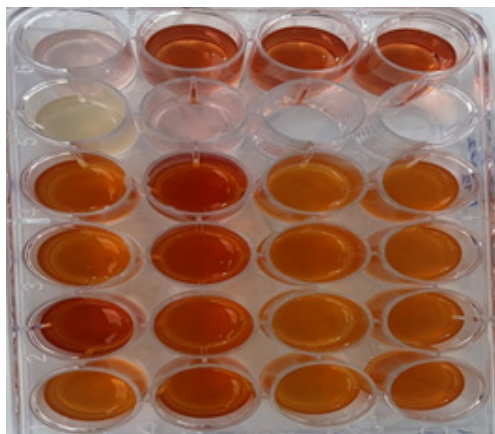


Fig. 2. An example plate of XTT reactions was conducted on five biofilms. The intensity of the orange color indicates the optical density (OD) and reflects the level of metabolic activity. A darker orange color indicates higher OD, indicating higher metabolic activity.

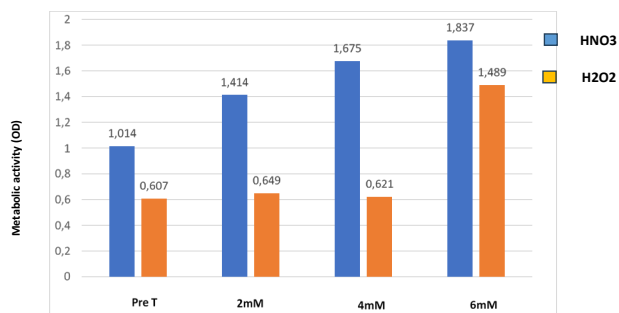


Fig. 3. The measured XTT absorbances for *Candida albicans* isolates were recorded before and after their exposure to H_2O_2 and HNO_3 , with pre-T, 2mM, 4mM and 6mM.

Table 2. The level of gene expression under the influence of H_2O_2 and HNO_3 .

Genes	H_2O_2			
	0 mM	2 mM	4 mM	6 mM
ALS1	1	1	1	1
EGR11	1	0	0	0
EXO1	1	0.9	0.9	0.9
HWP1	1	0.9	0.9	0.9
MLH1	1	1	1	0.9
CDR1	1	0	0	0
Genes	HNO_3			
	0 mM	2 mM	4 mM	6 mM
ALS1	1	39.6	3.7	217.2
ERG11	1	0.9	1	0.9
EXO1	1	0	0	0
HWP1	1	1	1	0.9
MLH1	1	1.1	1.1	1.1
CDR1	1	0	0	0

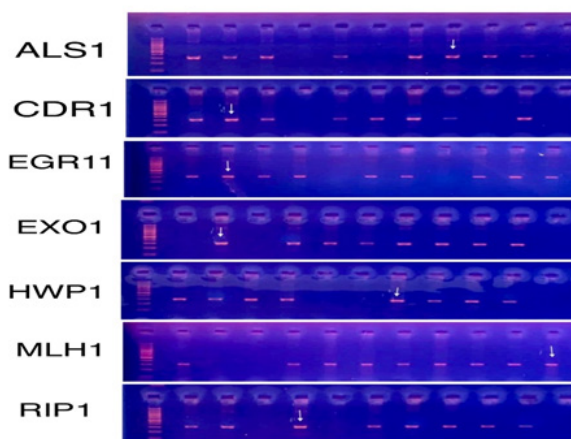


Fig. 4. Gel electrophoresis of the gene presence after amplification of the genes from cDNA templates.

superficial to life-threatening systemic infections. The biofilm formation and dimorphic capability of the yeast are essential in its pathogenicity and host responses. The ability of *Candida* to withstand stresses is of great concern. Previous studies indicated an active repair machinery of the yeast in response to breaks in its genetic materials [20]. The present study scanned transcriptional response in some concern genes to better understand the mechanism behind such repair and response cascade. This is through stressing *Candida albicans* with free radicals via different levels of H_2O_2 and HNO_3 .

Transcriptional analysis of the biofilm responding to different concentrations of oxidative and nitrosative stresses showed variable regulation of the genes selected for this study. Of concern, the ALS1 and MLH1 recorded a significant upregulation when treated with those stress compounds. ALS1 is known to have a vital role in biofilm formation and host interaction [21]. This indicates that a high level of HNO_3 can induce the yeast to form stronger biofilms, which may be a protective mechanism toward inhibition of the stress to enter cells of the yeast [22]. While MLH1 which is responsible for the repair of any mismatch that can be caused by the stress. It has been activated as per the results in this study with levels of H_2O_2 . This is an in-

dication of a known response of *Candida albicans* toward discrepancies and misconnections of nucleotides, arming the yeast with a great capability to withstand stresses and mutagenic radicals [23].

The study also investigated the role of these stresses in inducing genes related to antifungal resistance and lipid biosynthesis. The ERG11 gene is known to have a great role in ergosterol biosynthesis and antifungal resistance. Azoles specifically target the ergosterol in the fungal membrane. Our study showed down-regulation response of the ERG11 gene when stressed with oxidative and nitrosative stresses. This may be considered a possible mechanism of adaptation by halting the lipid biosynthesis to minimize lipid peroxidation effect of the free radicals.

The biofilms showed significant up-regulation of both ALS1 and MLH1 genes. The high baseline level of transcription may explain the biofilms' ability to counteract the mutagenic effects of acetaldehyde produced during their growth. The mean fold change in MLH1 expression was 39 times, while HWP1 exhibited an even higher fold change, reaching 149. The notable upregulation of MLH1 is particularly intriguing since studies on mammalian cells have indicated downregulation of this gene under hypoxic conditions, such as during infection [24]. The present study found a significant correlation between the expression rates of the two genes during biofilm formation.

Our study is the first to report that candidal biofilms can tolerate high levels of H₂O₂ and HNO₃. Interestingly, H₂O₂ appears to be less toxic to *C. albicans* biofilms compared to HNO₃. Recent publications [25–27] have extensively documented the impact of ethanol on biofilms. One detrimental effect of ethanol is its induction of the interdigitated phase in lipid membranes. Our data revealed a significant correlation (p -value=0.04) between the amount of ethanol and the expression level of the ERG11 gene, which plays a crucial role in ergosterol biosynthesis. These findings align with previous studies [28–30] that have demonstrated a relationship between ergosterol biosynthesis and ethanol levels in yeast growth environments.

Previous research has produced inconsistent results regarding the impact of *C. albicans*' inhibitory activity on iNOS. [31] reported a minor reduction in iNOS protein levels, but not mRNA levels, along with a significant decrease in iNOS enzymatic activity [26, 27]. The biofilm lifestyle provides *C. albicans* with a protective environment and serves as a source of persistent infection. We also aimed to investigate *C. albicans*' ability to protect and repair its genome from the mutagenic effects of H₂O₂ and HNO₃. This study aimed to investigate the effects of H₂O₂ and HNO₃ exposure on the growth, alcohol metabolism, and expression of azole resistance and DNA mismatch repair genes in *C. albicans* biofilms.

Nitric oxide (NO) production serves as a crucial defense mechanism against invading pathogens. Being a free radical, nitric oxide can readily diffuse through cell membranes and interact with various molecules, leading to different forms of cellular damage. The relationship between nitric oxide and specific human pathogens, including *C. albicans*, is intricate and requires further investigation. Nonetheless, existing evidence suggests that nitric oxide plays a significant role in controlling *C. albicans* infections. Studies have demonstrated that mice lacking nitric oxide production exhibit heightened susceptibility to *C. albicans* infections, as evidenced by increased organ load

[32].

Moreover, studies have shown that the elimination of *C. albicans* by murine saliva and macrophages, in specific instances, necessitates the presence of NOS2 [18, 19, 32]. *C. albicans* is a major fungal pathogen responsible for approximately 400,000 life-threatening systemic infections worldwide each year, primarily affecting severely immunocompromised patients. Innate immune cells play a vital role in combating fungal infections by utilizing a key mechanism that involves generating harmful ROS, including superoxide and hydrogen peroxide. Therefore, there is significant interest in unraveling the mechanisms employed by *C. albicans* to evade oxidative destruction by macrophages and neutrophils.

In recent years, significant progress has been made in understanding how *C. albicans* detect and respond to ROS. Notably, it has been observed that hydrogen peroxide stimulates the filamentation process in this polymorphic fungus and induces the expression of a distinct cell surface superoxide dismutase enzyme [33]. Furthermore, recent research has revealed that the combination of chemical stresses produced by phagocytes can actively impede the oxidative stress responses of *C. albicans* through a mechanism known as stress pathway interference. This review offers an up-to-date summary of our current knowledge regarding the role and regulation of oxidative stress responses in this noteworthy fungal pathogen that affects humans [34].

5. Conclusions

Understanding how *C. albicans* evades the immune system and takes advantage of weakened defenses is crucial for developing effective strategies to treat infections caused by this species, which is a significant cause of fungal infections in humans. The results obtained from this study offer valuable insights into the immune pathogenesis of *C. albicans* biofilm infections, enhancing our understanding of the underlying mechanisms involved. These findings contribute to a deeper comprehension of the subject matter.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed Consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors contributed equally in this research study.

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