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RESEARCH ARTICLE

Mediation of *Candida species* growth and virulence by the proinflammatory cytokine IL-6

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Interleukin-6 (IL-6) is a cytokine with pleiotropic effects that might also influence the virulence traits of some microorganisms, but its direct influence over *Candida spp*. is currently unknown. The objective of the study is to determine the influence of IL-6 (250 pg/ml) on the growth rate and biofilm formation of *C. albicans, C. parapsilosis, C. krusei, C. auris,* and *C. guilliermondii*, as well as to analyze the influence of this citokine on the expression of three virulence genes (*ALS3, HSP70, SAP2*), respectively on the germ tube formation ability of *C. albicans.* The influence of IL-6 on growth rate was assessed by incubating the fungal cells in presence of IL-6 for 48 hours and assessing the optical density of the samples at five timepoints. The biofilm production in presence of IL-6 was studied in microtiter plates, using crystal-violet assay, the intensity of biofilms being evaluated by spectrophotometry. The expression of *ALS3, HSP70,* and *SAP2* in *C. albicans* was studied by RT-PCR, reported to *ACT1* housekeeping gene. The germ-tube test was performed to assess the influence of IL-6 on the filamentation rate of *C. albicans.* All test results were normalized against control, without added IL-6. The results showed that IL-6 influences the growth rate of *C. albicans, C. parapsilosis, C. krusei, C. auris,* and *C. guilliermondii* in a time-dependent way. Also, IL-6 inhibited the formation of biofilms for *C. albicans* and *C. guilliermondii.* In *C. albicans* cells, IL-6 induced upregulation of *ALS3* and *HSP70*, while it down-regulated the *SAP2* gene. IL-6 did not influence the germ-tube formation in *C. albicans*. In conclusion, IL-6 might exert, *in vitro*, direct effects on the virulence traits of *Candida* spp., and its influence is dependent on the exposure time. Non-albicans *Candida* species presented particular responses to IL-6.

Keywords: growth rate, biofilms, *ALS3, HSP70, SAP2, Candida* spp.

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Introduction

Interleukin-6 (IL-6), a cytokine secreted by the T cells, influences a wide range of cellular functions. Elevated serum levels of IL-6 correlate, with the onset of type 2 diabetes, because of its ability to promote the onset of insulin resistance and, also, because of its pro-inflammatory effects [1]. During inflammation, IL-6 can block the apoptosis process of the human cells, keeping them alive in environments otherwise considered toxic [2]. Distortions in the activity of IL-6 promote the onset of chronic disease and even cancer [3].

The genus *Candida* is polyphyletic and includes numerous species, many incompletely studied. Although *C. albicans* is a frequently isolated and well-characterized member of the genus, non-albicans species are causing an increasing number of infections [4,5]. *C. parapsilosis,* for example, can be the etiologic agent of invasive infections (with a mortality rate ranging between 4-45%), endocarditis and rarely otomycosis, vulvovaginitis, and urinary tract infections [6]. The newly emergent species *C. auris* can cause hard-to-treat infections, especially in healthcare facilities. Because of its intrinsic resistance to commonly used disinfectants and because of its innate and acquired resistance to antifungal agents, *C. auris* is a new nosocomial threat [7]. Although *C. guilliermondii* is a rare species from the non-albicans group, it can cause chronic onychomycosis, periodontitis, sepsis, acute osteomyelitis, endocarditis, skin infections, silent candidiasis, dentoalveolar abscesses, septic arthritis, or cellulite, mainly in immunocompromised patients [8].

Candida infections are the result of complex microorganism-host interactions [9]. Because IL-6 is a cytokine involved in the immune response of the host, it might directly influence the yeast cells' behavior. The study aims to analyze, *in vitro,* the effects of IL-6 on the growth rate and biofilm production of five *Candida* species: *C. albicans*, *C. parapsilosis, C. krusei*, *C. auris,* and *C. guilliermondii*, as well as to analyze the influence of IL-6 on the expression of three virulence genes (*ALS3*, *HSP70,* and *SAP2*) and the germ tube formation ability of *C. albicans*. The novelty of the study consists in studying the direct effect of IL-6 on *Candida* spp., as well as in including insufficiently studied non-albicans species. Working hypothesis: IL-6, as a pro-inflammatory cytokine, can directly influence yeast growth and virulence.

Material and method

To assess the influence of IL-6 (human Interleukin-6, Sigma-Aldrich, MO, USA) on the fungal growth and virulence, *Candida albicans* ATCC 90028, *Candida auris* CBS 10913, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, and *Candida guilliermondii* IC184 (Cantacuzino Institute, Romania) reference strains were

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used. The yeasts were cultured and checked for purity on Sabouraud agar (Oxoid, U.K.), then subcultured on Sabouraud dextrose broth (SDB) (Oxoid, U.K.), (for growth rate and gene expression) or RPMI medium (Oxoid Limited, UK) buffered with MOPS (for biofilm assay), either in the absence (control) or in the presence of recombinant IL-6. The lyophilized IL-6 was reconstituted, according to the manufacturer's instructions, to achieve *in vitro* physiological final value of 250 pg/ml, comparable with achievable human serum concentrations during infections (range between 0 and 552.6 pg/ml) [10]. The experiments were performed in triplicate, based on previously published protocols [11,12]. As follows, the used methods are described shortly.

For assessment of the growth rate, approximately 10^3 CFU of each *Candida* spp. were inoculated in 10 ml SDB and incubated at 35°C for 48h in a shaking water bath, in absence or presence of 250 pg/ml IL-6, followed by a sequential reading of the inoculum optical density (OD600) at different time points (after 6, 9, 12, 24, and 48 hours of incubation). For biofilm formation, approximately 10^4 CFU of each *Candida* spp. were incubated in 100 µl RPMI-1640 culture medium, at 35°C for 24h, in the wells of a 96-well polystyrene plate, in the absence or presence of 250 pg/ml IL-6. The biofilm production was assessed by crystal-violet assay (coloration of adherent cells with crystal violet, washing, resuspension in 100 µl acetic acid). The OD620 of the resulted solutions were read by spectrophotometry in a Dynex DSX Automated ELISA analyzer (Dynex Technologies, Inc., USA). The growth rate and the biofilm production in presence of IL-6 were calculated by dividing the mean OD value of the IL-6 treated samples with the mean OD value of the control (Δ-Index) for each *Candida* species. Δ-Index values ≤ 0.75 and ≥ 1.25 were considered significant for inhibition, respectively stimulation, while values between 0.75 and 1.25 were considered indifferent on the growth rate or biofilm formation (variability of +/- 25% was attributed to chance).

To study the influence of IL-6 on the expression of virulence genes, 10^4 CFU of *C. albicans* cells were incubated in 1.5 ml SDB at 35°C for 3h, in the absence or presence of 250 pg/mL IL-6. After cell lysis (freeze-thaw and lyticase treatment), total RNA was extracted (IndiSpin Pathogen Kit, Indical Bioscience, Germany), treated with DNase I (Thermo Scientific, UK), and reverse transcripted using GoScript Reverse Transcription System (Promega, USA). The expression of the *ALS3*, *HSP70,* and *SAP2* genes was appreciated by Real-Time PCR (GoTaq PCR Mastermix, Promega, Madison, WI, USA), using specific primers for *C. albicans* [11,12]. The amplification was performed in the Applied Biosystems Quantstudio 5 PCR system. The gene expression was calculated by comparing the mean cycle threshold (Ct) value of the samples with the mean Ct value of the control and normalized against corresponding Ct for housekeeping gene *ACT1*, resulting in a fold-change (Fc) value. Fc values <0.75 indicated down-regulation of the gene, while Fc values > 1.25 indicated upregulation. A 0.25 variation of the Fc value was attributed to chance.

The filamentation rate was studied by incubating equal amounts of *C. albicans* inoculum and blood plasma, without or with added IL-6 (250 pg/ml). The percentage of yeast cells that produced germ tubes was evaluated using a brightfield microscope, both for control and IL-6 treated samples.

The data were analyzed in GraphPad InStat3, with an alpha value set at 0.05.

Results

In the presence of IL-6, *C. albicans* growth rate was slightly inhibited at 6h and 9h and enhanced after 12h of incubation, without any differences at 24h and 48h of incubation compared to control. The growth of *C. parapsilosis*, on the other hand, was inhibited at 9h of incubation, stimulated after 12 h, and not influenced (above the chosen significance threshold) in the following time points. The growth rate of *C. krusei* was not influenced by IL-6 in the first 12h of incubation but was inhibited after 24h and 48h of incubation. *C. auris* growth rate was initially stimulated (after 6h of incubation), then inhibited (after 9h and 12h of incubation), with no significant effects after 24 and 48 hours. The growth rate of *C. guilliermondii* was not affected by IL-6 in the first 9 hours, but it was enhanced at 12h and 24h (Figure 1).

IL-6 in concentrations of 250 pg/ml inhibited the production of biofilms by *C. albicans* and *C. parapsilosis* and it did not influence the formation of biofilms for *C. krusei*, *C. auris,* and *C. guilliermondii* (the Δ-Index values did not exceed the significance threshold) (Figure 2).

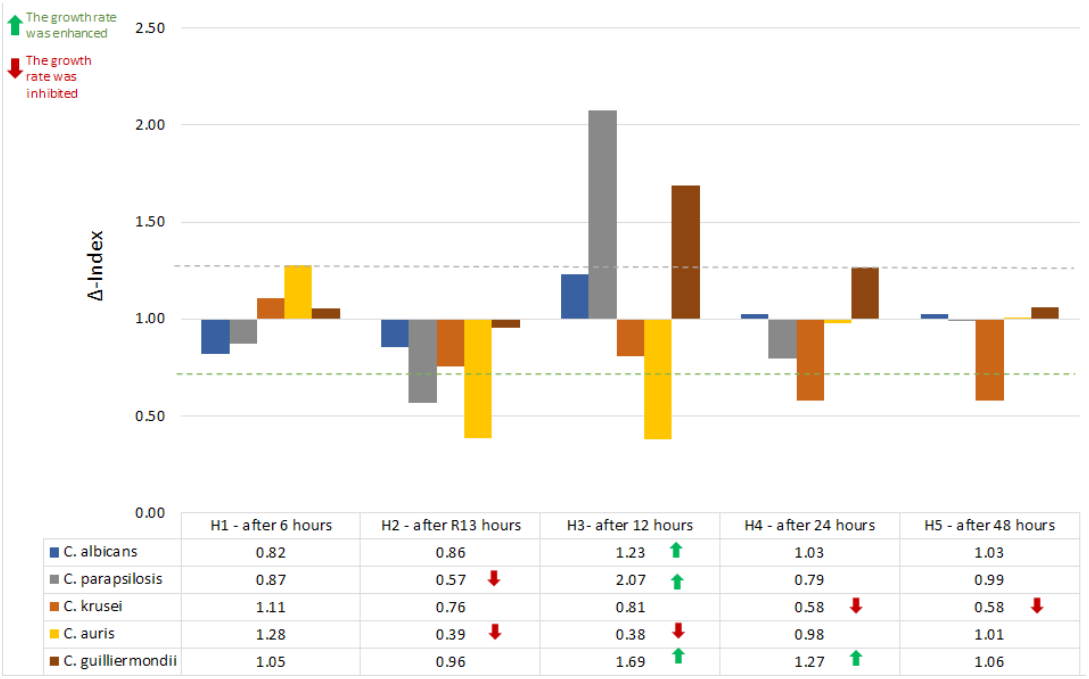
IL-6 (250 pg/mL) induced overexpression of *ALS3* and *HSP70* genes, and underexpression of *SAP2* gene in *C. albicans* (Figure 3).

Germ tubes were found in 93% of *C. albicans* cells incubated in presence of 250 pg/mL IL-6 (Figure 4). There were no significant differences in the germ tube formation rate compared with control ($p < 0.05$), where the filamentation rate was 92%.

Discussions

IL-6 has pleiotropic effects, many of them still to be discovered. The present study proves that, *in vitro*, IL-6 can express its pleiotropic effect on the growth rate, biofilm formation ability of some *Candida* species, or on the expression of specific *C. albicans* virulence genes.

During cell invasion, *C. albicans* promotes the secretion of IL-1α, IL-6, IL-8, and TNF-α, and more virulent strains were found to determine higher humoral inflammatory responses [13]. This study emphasizes the fact that different *Candida* spp. respond in particular ways to pure IL-6, apart from the other immunomodulatory factors of the host. As the majority of the published studies focus on *C. albicans*, some species of the non-albicans group are often overlooked.



	H1 - after 6 hours	H2 - after R13 hours	H3- after 12 hours	H4 - after 24 hours	H5 - after 48 hours
C. albicans	0.82	0.86	1.23 ↑	1.03	1.03
C. parapsilosis	0.87	0.57 ↓	2.07 ↑	0.79	0.99
C. krusei	1.11	0.76	0.81	0.58 ↓	0.58 ↓
C. auris	1.28	0.39 ↓	0.38 ↓	0.98	1.01
C. guilliermondii	1.05	0.96	1.69 ↑	1.27 ↑	1.06

Fig. 1. The influence of IL-6 on the growth rate of five *Candida* spp.

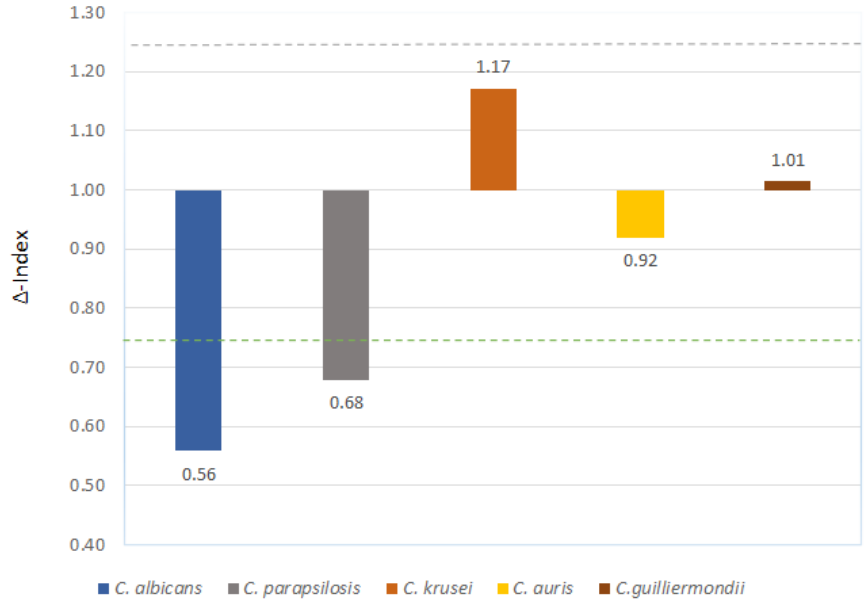


Fig. 2. The influence of IL-6 on the ability of *C. albicans*, *C. parapsilosis*, *C. krusei*, *C. auris*, and *C. guilliermondii* to form biofilms

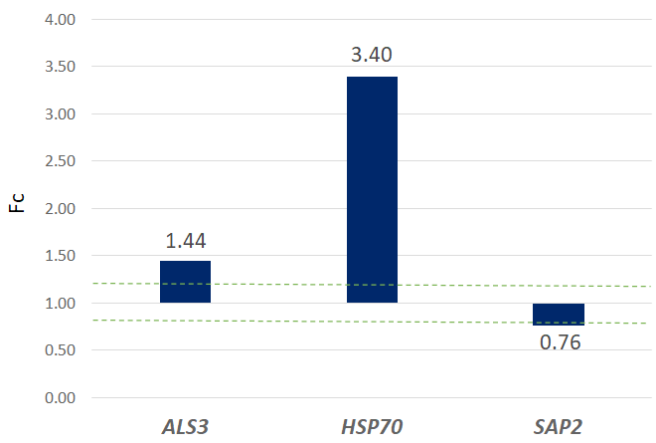


Fig. 3. The influence of IL-6 on the expression of *ALS3*, *HSP70*, and *SAP2* in *C. albicans*

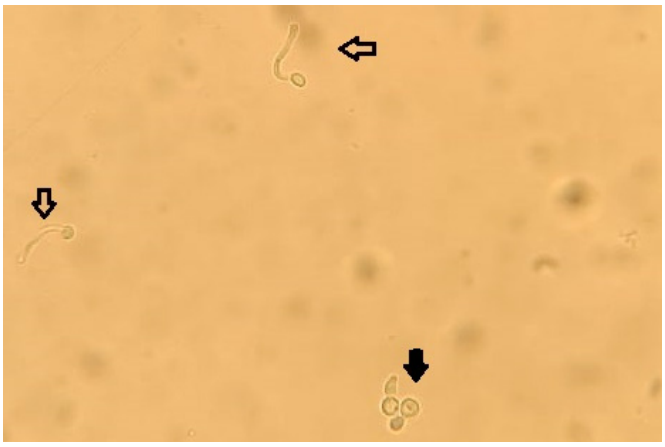


Fig. 4. Representative image showing *C. albicans* germ tube formation in the presence of IL-6. Outlined arrows – cells with germ tubes; full arrow - cells without germ tubes

Although the role of IL-6 in the immune response is widely studied, little is known about its direct influence on microorganisms. In a study conducted by Engelsöy et al. in 2019, IL-6 stimulated the growth of *Escherichia coli* and upregulated the expression of genes involved in the acquisition of iron, a virulence trait of *E. coli*, thus influencing its virulence [14]. IL-6 might modulate the virulence traits of some microorganisms, but to our knowledge, this is the first study that analyzes the effects of IL-6 on the growth rate and biofilm formation of *C. albicans*, *C. parapsilosis, C. krusei*, *C. auris,* and *C. guilliermondii*.

In a study conducted on mice, Ahmadi et. al found out that during candidemia, *C. albicans* can disrupt the cytokine network, by affecting the IFN-γ/IL-4 ratio and promoting the secretion of IL-10, TNF-α, and TGF-β [15]. Cytokines can induce the immune response against *C. albicans* and promote phagocytosis [16], thus affecting *Candida* spp. via indirect pathways. Our study highlights the fact that IL-6 can also directly influence the growth rate of *Candida* spp., but the inhibition/stimulation effects on the growth rate were not persistent in time. For example, *C. auris* growth was initially enhanced by IL-6, but then it decreased and ultimately, no differences were observed, compared with the control samples. The stimulating effects ceased in time, probably due to adaptative mechanisms. After 48 hours, only the growth rate of *C. krusei* was still inhibited by IL-6. IL-6 may act as a stress factor against *Candida* cells, but *Candida* spp. possess multiple stress adaptative mechanisms. These mechanisms are species-related [17], which might explain why in our study the growth rates and biofilm formation ability of *C .albicans*, *C. parapsilosis*, *C. krusei*, *C. auris*, and *C. guilliermondii* were differently influenced by IL-6.

To invade various host niches, *Candida* spp. cells rely on the expression of multiple virulence factors: adhesion proteins (like Als3), hydrolytic enzymes (like Saps, secreted aspartic proteinases), reversible morphological transitions, production of biofilms, and many others [9]. Biofilms are reservoirs of *Candida* spp. cells that can detach from biofilms, causing disseminated infections. The cell density inside of a biofilm provides a physical barrier, and cells organized in biofilms are more resistant to antifungal drugs or immune-related molecules [18]. The present study highlights the inhibitory effect of IL-6 on biofilm development for *C. albicans* and *C. parapsilosis*, despite the growth spike at 12h incubation time of the latter species.

Leukocytes have a differential production of cytokines against cells organized in biofilms, compared with planktonic cells [19]. Chandra et al. showed that peripheral blood mononuclear cells secrete lower levels of IL-6, IL-10, TNF-α, MCP-1, I-309, and MIP1β against *C. albicans* cells and increased levels of IL-1β. For these experiments, the peripheral blood mononuclear cells were allowed to act on biofilms at the adhesion phase [20]. The stage of the biofilm is important because macrophages have specific effects on the different stages of biofilm maturation. Biofilms formed in the presence of macrophages are less thick, and in the first stages of biofilm production, the macrophages can phagocyte *C. albicans*. Interestingly, when macrophages interact with mature biofilms, they are increasing the biofilm formation [21]. Yeast cells organized in biofilms might have different responses to cytokines than planktonic cells. Also, cytokines act differently, depending on the stage of the biofilm maturation. In the present study, as the samples were incubated in the presence of IL-6 for 24 hours, IL-6 was allowed to act from the early stages of the biofilm production. Thus, the inhibitory effect of IL-6 might be correlated with the initial stage of biofilm production and the young age of yeast cells.

Conversely, the cytokine response to biofilms formed by *Candida* spp. cells varies among the species that form the biofilm [22]. As biofilms are hard to eradicate and infections occur as a result of the complex interplay between *Candida* spp. and the host immune system [9], the ability of IL-6 to inhibit *in vivo* biofilm production may be species-specific. Even if some cytokines might inhibit the production of biofilms, the structural characteristics of biofilm shelter the microbial cells from eradication [23].

Cell adhesion and biofilm formation capability are species-related, and several genes take part in this process. *ALS3* gene encodes an agglutin-like sequence proteins involved in cell adhesion. Als3 can bind to human laminin, fibronectin, collagen, E-cadherin, N-cadherin, and fibrinogen [24]. In the present study, IL-6 induced the overexpression of *ALS3*, while it inhibited the biofilm production for *C. albicans*. Probably, following biofilm inhibition, *C. albicans* responded by overexpressing genes involved in biofilm production, as a compensation mechanism.

Other genetic factors are also important for *Candida* spp. virulence. For example, *C. albicans* harbors 10 SAP genes, encoding preproenzymes (60-200 amino acids longer than the enzymes), some of which are secreted into the extracellular environment (Sap1-8) and some GPI-anchored (Sap9-10). The expression of SAP genes is strain and environment-dependent [25]. *SAP2* (the gene that encodes Sap2, secreted aspartic proteinase 2) overexpression is thought to be an early biomarker of hypervirulent *C. albicans* strains. Through direct or indirect mechanisms, Sap2 recruits neutrophils at the site of the infection and promotes the secretion of interleukins. On the other hand, Saps can activate caspase-1, an enzyme that can inhibit the secretion of some interleukins like IL-1β and IL-18 in epithelial cells [26]. In our study, IL-6 downregulated the expression of *SAP2*, thus the production of *Candida*-secreted proteinases might be directly affected by this cytokine.

As a response to stress conditions, *Candida* can adapt by producing *HSP70*, an ATP-dependent chaperone protein, located in the cytoplasm of multiple eukaryotic cells, with important roles in the adaptation to stress (by influencing the MAPK, Ras1-cAMP-PKA, and calcium-calcineurin pathways and being involved in cell cycle control signaling) [27,28]. In a study conducted by Dockladny et al.

on laboratory animals, overexpression of *HSP70* induced the production of IL-6 [29]. We showed that exogenously supplemented IL-6 can induce overexpression of *HSP70* in *C. albicans*. Because *HSP70* is involved in the stress response, the results are in concordance with the working hypothesis.

To invade the underlying substratum, *C. albicans* can form hyphae, a form of anisotropic growth where the volume of the cell extends along a polarized axis. This starts with the generation of a germ tube, that will extend to form the filamentous structures [30]. The filamentation process is also part of biofilm production. Fungal cells start their filamentation during the biofilm initiation stage, and true hyphae are formed as the biofilm matures [31]. In this study, IL-6 did not inhibit the germ tube formation in *C. albicans*, but it inhibited the biofilm production, probably through other mechanisms.

The search on PubMed for different terms combinations ("IL-6 candida growth rate [title]" [32], "IL-6 candida biofilms [title]"[33], "IL-6 candida virulence [title]" [34]), returned no results. The majority of publishes paper focuses on the role that IL-6 plays in the immune response of the host, and to our knowledge, this one the first studies to analyze the direct effects of IL-6 on *Candida* spp. virulence.

Conclusions

Even though IL-6 exhibits pleiomorphic effects *in vivo*, the current study emphasizes the fact that IL-6 can directly influence the virulence traits of some *Candida* spp., *in vitro*. IL-6 can affect the growth rate of some *Candida* spp. (especially after 12 hours of incubation), but its influence over the fungal growth seizes in time. Also, IL-6 can inhibit the biofilm production for *C. albicans* and *C. guilliermondii*, and it affects the expression of *ALS3*, *HSP70*, and *SAP2* virulence genes in *C. albicans*.

As, *in vivo*, the occurrence of infection with *Candida* spp. is a partial win in a long-lasting battle between the immune system and the fungal cells, the current study helps to better understand the complex pathogen-host molecular relationships.

Authors contribution

C.N.C. - Conceptualization, Data curation, Investigation, Methodology, Validation, Formal Analysis, Writing – original draft, Writing – review & editing;
I.B.K. - Formal Analysis, Investigation, Methodology, Validation, Resources, Writing – review & editing;
F.T. - Formal Analysis, Investigation, Methodology, Resources, Writing – review & editing;
B.T. - Formal Analysis, Investigation, Methodology, Writing – review & editing;
M.M. - Formal Analysis, Investigation, Methodology, Validation, Resources;
A.M. - Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. Funding acquisition, Supervision.

Conflicts of Interest

The authors declare no conflict of interest.

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