RESEARCH ARTICLE

# An alternative respiratory pathway on *Candida krusei*: implications on susceptibility profile and oxidative stress

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## Abstract

Our aim was to detect the presence of an alternative oxidase (AOX) in *Candida krusei* clinical strains and its influence on fluconazole susceptibility and in reactive oxygen species (ROS) production. *Candida krusei* clinical isolates were tested to evaluate the presence of AOX. *Debaromyces hansenii* 2968 (AOX positive) and *Saccharomyces cerevisiae* BY4742 (AOX negative) were used as control strains. Measurements of oxygen consumption were performed in the presence of 1 mM KCN, an inhibitor of the classical respiratory chain, and 5 mM salicylhydroxamic acid (SHAM). AOX expression was monitored by Western blotting using an AOX monoclonal antibody. Interactions between fluconazole and SHAM were performed using checkerboard assay. ROS production was evaluated in the presence of SHAM plus fluconazole, $H_2O_2$, menadione, or plumbagin. AOX was present in all *C. krusei* tested. The combination of fluconazole with SHAM resulted in an indifferent effect. In the presence of SHAM, the treatment with ROS inductors or fluconazole increased ROS production, except in the AOX-negative strain. An alternative respiratory pathway resistant to cyanide is described for the first time as a characteristic of *C. krusei* species. This AOX is unrelated to fluconazole resistance; however, it protects *C. krusei* from oxidative stress.

## Introduction

Over the past few decades, authors have documented increases in the rate of candidemia by non-*albicans* species of *Candida*, such as *Candida krusei*, especially in critically ill and immunocompromised patients with hematologic malignancies (Hachem *et al.*, 2008).

Fluconazole is often used in the prophylaxis and treatment of candidemia and is the first-line therapy for this condition (Pappas *et al.*, 2009). Among non-*albicans* species, *C. krusei* is the only species that is predictably fluconazole resistant (Oxman *et al.*, 2010). Antifungal drug tolerance can also be modulated by metabolic adaptability mechanisms, including alterations in the respiratory mitochondrial pathway (Chamilos *et al.*, 2006; Brun *et al.*, 2003). This fact had been previously assessed in *C. glabrata*, *C. albicans*, and *C. parapsilosis* regarding its influence on fluconazole and caspofungin (Brun *et al.*, 2003; Chamilos *et al.*, 2006; Yan *et al.*, 2009).

In eukaryotic organisms, the energy necessary for growth, development, reproduction, and stress response is acquired through the ATP synthesized during mitochondrial respiration, where cytochrome *c* oxidase acts as a terminal oxidase in the reception of electrons and converting oxygen into water. Within the mitochondrial respiratory chain, another route mediated by the alternative oxidase (AOX; a mitochondrial enzyme) can be found in plants, in certain protozoa and fungi (Moore & Siedow, 1991; Helmerhorst *et al.*, 2002, 2005; Veiga *et al.*, 2003a, b). This AOX is insensitive to cytochrome pathway inhibitors, such as antimicin A or cyanide, but is specifically inhibited by salicylhydroxamic acid (SHAM) and confers a cyanide-resistant respiration through an alternative respiratory pathway (ARP) to such organisms (Moore

& Siedow, 1991). The AOX is located on the matrix side of the inner mitochondrial membrane and plays an important role in susceptibility to azole antifungals in *C. albicans* (Yan *et al.*, 2009). The alternative respiratory chain can be activated by stress situations like the presence of antifungals or oxidative inductors, thus leading to drug tolerance and to the reduction in generation of intracellular reactive oxygen species (ROS; Cannon *et al.*, 2007).

In an attempt to explain the contribution of mitochondrial respiration in the intrinsic fluconazole resistance displayed by *C. krusei*, we assessed the existence of an ARP and its influence upon fluconazole resistance and tolerance to oxidative stress in *C. krusei*.

## Materials and methods

### Drugs and chemicals

Fluconazole was obtained from Pfizer (Groton, CT), and stock solutions were prepared according to CLSI M27 A3 protocol and maintained at −70 °C until use (CLSI, 2008). Stock solutions of SHAM (200 mM; Sigma-Aldrich, Germany) and potassium cyanide (KCN, 1 M; Sigma) were prepared in DMSO (Sigma) and distilled water, respectively. Hydrogen peroxide ($H_2O_2$; 30% v/v) was obtained from Merck. Plumbagin and menadione were obtained from Sigma, and stock solutions (100 mM) were prepared in 95% ethanol. Dihydrorhodamine 123 (DHR123) was obtained from Molecular Probes (Eugene, OR).

### Strains and culture conditions

A total of 25 *C. krusei* (nine from respiratory secretions, one from blood, 11 vaginal, three fecal, and the type strain ATCC 6258 from the American Type Culture Collection), previously identified using VITEK II system (BioMérieux, Paris, France), all resistant to fluconazole were used in this study. *Candida albicans* SC5314 strain was used as positive control for the presence of AOX-specific protein. The strains were grown in Sabouraud dextrose broth (Difco) at 30 °C, in an orbital shaker at 150 r.p.m. until late exponential growth phase ($OD_{640}$ 1.5). *Debaromyces hansenii* IGC2968 (LGC Standards S.L. U., Barcelona, Spain) was used as the positive control for the presence of an ARP, assessed by oxygen consumption. *Saccharomyces cerevisiae* BY4742 (EUROSCARF, Frankfurt, Germany) was used throughout the study as the negative control for the presence of the AOX assessed by oxygen consumption, immunoblotting, and ROS production. These control strains were grown in a mineral medium with vitamins and 2% (w/v) glucose until stationary growth phase ($OD_{640}$ 2.5 and 3, respectively; Veiga *et al.*, 2003a, b).

### Alternative respiratory pathway

Late exponential phase cultures were centrifuged (18 000 ***g***) for 4 min at 4 °C and washed twice with cold sterile water. An small amount (1.5 g wet weight) of pellet was resuspended in 100 mL of 50 mM phosphate-buffered saline, pH 6.0 with 0.1% cicloheximide (Sigma, St. Louis, MO), and 6 mL of suspension was incubated in a small reactor at 28 °C. The $O_2$ consumption was continuously measured with a Clark-type electrode YSI model 5775 (YSI Incorporated, Yellow Springs, OH) after the addition of 200 μL of 1.55 M glucose (Difco Laboratories, Detroit, MI), 3.2 mM of KCN, and/or 3.2 mM of SHAM (Sigma-Aldrich). The presence of an AOX was considered whenever the oxygen consumption pattern was resistant to KCN, but sensitive to SHAM.

### Checkerboard microdilution assay

Checkerboard assays were performed with all *C. krusei* strains, in the presence of fluconazole and SHAM, using the protocol described in the Clinical Microbiology Procedures Handbook (Moody, 1991). The concentration range used was 0.125–64 μg fluconazole $mL^{-1}$ and 0.6–20 mM for SHAM. The minimal inhibitory concentrations (MIC) of each compound were determined according to CLSI M27 A3 and S3 protocol in RPMI 1640 (Sigma; CLSI, 2008). Fractional inhibitory concentration of fluconazole ($FIC_A$) was calculated as the MIC of fluconazole in combination/MIC of fluconazole alone, and $FIC_B$ is the MIC of SHAM in combination/MIC of SHAM alone. The fractional inhibitory index (FIX) was calculated as follows: $FIX = FIC_A + FIC_B$. The interpretation of FIX was as recommended: $\leq 0.5$, synergistic effect; $> 0.5$ to $< 4.0$, no interaction; $\geq 4.0$, antagonistic effect (Moody, 1991).

### Assessment of intracellular ROS accumulation

Intracellular ROS accumulation was estimated to study the influence of an AOX upon *C. krusei* oxidative stress tolerance. After being washed and resuspended in PBS, $10^7$ cells $mL^{-1}$ were treated with ROS inductors such as $H_2O_2$ (0.4 mM), menadione (0.5 mM), and plumbagin (0.003 mM) for 15, 30, and 60 min, at 35 °C under 150 r.p.m. Yeast cells were also treated with fluconazole and voriconazole at MIC concentrations for 1 h. All the treatments were repeated following the preincubation of yeast cells with 3.2 mM of SHAM, an inhibitor of the AOX, during 30 min. Control cells were treated with a

DMSO concentration similar to that used in cell samples pretreated with SHAM. The cells were then collected by centrifugation and resuspended in PBS. Free intracellular ROS were detected with 15 μg dihydrorhodamine 123 $mL^{-1}$ (Molecular Probes; Almeida *et al.*, 2007; Mesquita *et al.*, 2010). Cells were incubated during 90 min at 30 °C in the dark, washed in PBS, and 5 μL was placed in a glass slide and overlapped with vectashield fluorescence mounting media (Vector Laboratories, Peterborough, UK). The stained suspensions were visualized under epifluorescence microscopy (40×; Olympus BX61). In each condition, a minimum of 500 cells from three different replicates were counted, and the percentage of stained cells (cells displaying ROS) was determined.

### Chronological life span

Overnight cultures of *C. krusei* strains were incubated in YPD broth until stationary phase (day 0 of chronological life span; Mesquita *et al.*, 2010). Cultures were treated without (control) and with 3.2 mM SHAM, 0.25 mM, and 0.5 mM of menadione alone, and in combination with 3.2 mM of SHAM. Survival was assessed daily by counting colony-forming units (CFUs) from cultures aliquots in YPD agar plates beginning at day 0 (when viability was considered to be 100%).

### Preparation of mitochondrial extracts

Mitochondrial extracts were isolated from *S. cerevisiae*, *Candida albicans* SC5314, and *C. krusei*. Cells were grown to stationary phase, harvested, and cell wall was digested with zymolyase buffer [2 M sorbitol-D, phosphate buffer 1 M (pH 7.5), zymolyase 20 000 U, 125 mM β-mercaptoethanol, 0.5 M EDTA] at 30°C for 1 h. Protoplasts were disrupted with lysis buffer [sorbitol-D 0.5 M, Tris 20 mM, EDTA 1 mM, and 2.85 mM phenylmethanesulphonyl fluoride (PMSF)] using a Potter homogenizator. Mitochondrial extracts were separated, washed by high-speed centrifugation at 16 420 ***g*** for 15 min at 4°C (Beckman Coulter, JA-25.50 Rotor), and resuspended in sorbitol buffer (0.5 sorbitol-D, 5 mM EDTA, 50 mM Tris). Protein concentration was determined by Bradford method (Kruger, 1994), and protein aliquots of mitochondrial extracts (40 μg) were stored at −20°C.

### Immunoblotting

Mitochondrial protein extracts were resolved on a 12% SDS gel and transferred to a nitrocellulose membrane. The membrane was blocked with phosphate-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% skim milk, followed by incubation with antibodies against AOX proteins (1 : 100) of *Sauromatum guttatum* (Agrisera AB, Vännas, Sweden) in TBST containing 1% skim milk. After washing with TBS, the membranes were incubated with the respective secondary antibody, HRP-conjugated anti-mouse IgG at a dilution of 1 : 5000, and detected by enhanced chemiluminescence.

### Statistical analysis

All experiments were performed in triplicate. Mean values were compared using Student's *t*-test whenever indicated. A $P$ value $< 0.05$ was considered statistically significant.

## Results

### Presence of a cyanide-resistant respiration pathway in *C. krusei*

To investigate whether an ARP was present in clinical isolates of *C. krusei*, measurements of $O_2$ consumption were performed in the presence of 3.2 mM of cyanide, an inhibitor of the classical respiratory chain (by inhibiting the cytochrome *c* oxidase complex), and SHAM, an inhibitor of the ARP (by inhibiting the AOX). In *C. krusei* strains, $O_2$ consumption stopped only after the addition of SHAM (Fig. 1a, representative example). This observation was made for all the 24 *C. krusei* clinical isolates, with ATCC 6258 type strain and with the positive AOX control strain *Debaromyces hansenii* 2968 (Fig. 1b). In *Saccharomyces cerevisiae* BY4742, the negative AOX control strain, the oxygen consumption stopped soon after the addition of cyanide (Fig. 1c). The results indicate that *C. krusei* cells have a cyanide-resistant respiration pathway promoted by the presence of an AOX.

### AOX expression

Mitochondrial preparations were immunoblotted with a monoclonal antibody raised against *S. guttatum* AOX, but also recognize *C. albicans* AOX (Huh & Kang, 1999). In all *C. krusei* clinical isolates, mitochondrial AOX was clearly detectable as well as in *C. albicans* SC5314 strain (Fig. 2). Mitochondria isolated from the negative control strain, *S. cerevisiae* BY4742, showed no reactivity with AOX antiserum (Fig. 2).

### Fluconazole–SHAM combination did not influence fluconazole activity

To study the influence of the ARP upon fluconazole resistance by *C. krusei*, we explored the *in vitro* combination

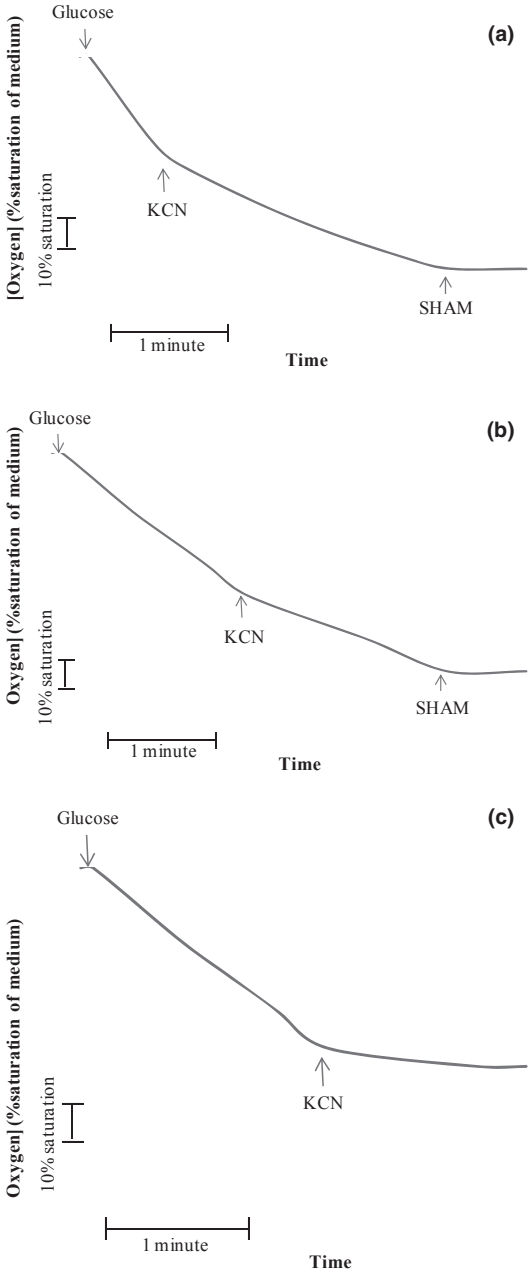


**Fig. 1.** Effect of KCN and SHAM upon oxygen consumption by *Candida krusei* clinical strain (representative example) (a), *Debaryomyces hansenii* (AOX positive control strain) (b), and *Saccharomyces cerevisiae* (AOX negative control strain) (c). Oxygen consumption was measured with an oxygen electrode at 28 °C. Where indicated (arrows), glucose (0.051 mM), KCN (3.2 mM), and SHAM (3.2 mM) were added.

of fluconazole and SHAM using the checkerboard methodology. SHAM produced no impairment of antifungal activity even at a concentration of 10 mM. The addition of SHAM did not change MIC values for fluconazole, resulting in an indifferent effect in all *C. krusei* isolates.

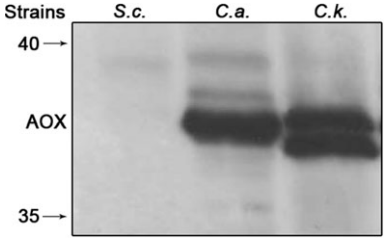


**Fig. 2.** Representative example of the presence of an AOX in *Candida krusei*. Immunoblot analysis of AOX levels in 40 μg of mitochondrial extracts of *Saccharomyces cerevisiae* (*S.c.*), *Candida albicans* SC5314 (*C.a.*), and *Candida krusei* (*C.k.*).

### The presence of an alternative respiratory pathway in *C. krusei* relates to reduced ROS accumulation

To assess the influence of the ARP upon oxidative stress response, we measured intracellular ROS accumulation with and without SHAM, using DHR123. This fluorochrome enters the yeast cell as a freely permeable dye, which is converted to rhodamine 123 and subsequently localized in the mitochondria. The conversion from the nonfluorescent to the fluorescent molecule is entirely dependent upon the presence of oxidation products. After treatment with the ROS-inducing agents, $H_2O_2$, plumbagin, menadione, and with azoles, the percentage of stained cells (cells with ROS accumulation) was calculated and compared with values displayed by nontreated cells, in the presence and absence of SHAM (3.2 mM). No significant differences regarding ROS accumulation were observed with the AOX-negative strain, *S. cerevisiae* BY4742, in the presence or in the absence of SHAM (Fig. 3). In contrast, after treatment with ROS-inducing agents, *C. krusei* cells treated with SHAM produced significantly ($P < 0.001$) more endogenous ROS than the cells with the unblocked ARP (without SHAM; Fig. 3). Significant differences in ROS production ($P = 0.008$) were also accomplished with fluconazole (Fig. 3). All *C. krusei*-tested strains displayed similar results.

Chronological life span decreased in *C. krusei* strains incubated with menadione when compared with untreated cultures (Fig. 4). The viability of *C. krusei* strains reduced significantly after 3 days of incubating the cultures with menadione plus SHAM (Fig. 4).

## Discussion

Several authors have stressed the relevance of the mitochondrial respiration and its influence upon metabolic behavior, stress environment adaptability, and antifungal drug tolerance (Brun *et al.*, 2003; Chamilos *et al.*, 2006; Yan *et al.*, 2009; Li *et al.*, 2011). A mitochondrial ARP

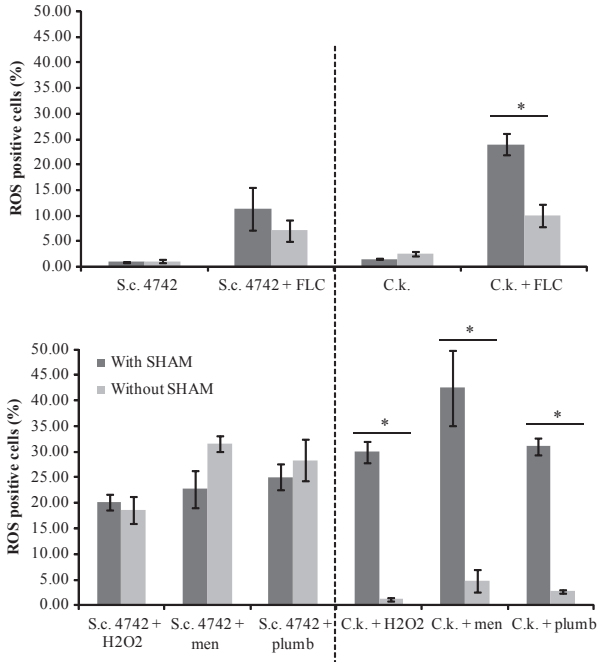


**Fig. 3.** Effect of fluconazole (FLC) and oxidative inductors (menadione – Men; plumbagin – Plumb; hydrogen peroxide – $H_2O_2$) upon intracellular ROS accumulation by a clinical *Candida krusei* (*C.k.*) strain (representative example) and a negative control strain *Saccharomyces cerevisiae* (*S.c.*), with or without the addition of SHAM. ROS accumulation was calculated and expressed as the percentage of DHR123-stained cells (*$P < 0.05$).

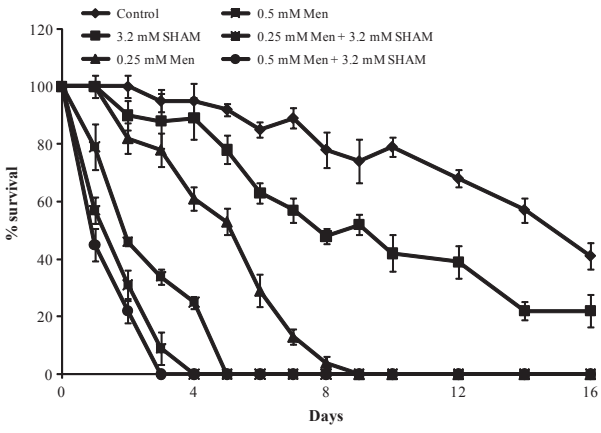


**Fig. 4.** Chronological life span of a *Candida krusei* strain. Strains were incubated without (control) and with 3.2 mM SHAM, 0.25 mM, and 0.5 mM of menadione (Men) alone and in combination with 3.2 mM of SHAM. Survival was assessed daily by counting colony-forming units (CFUs) from cultures aliquots in YPD agar plates beginning at day 0 (when viability was considered to be 100%).

(cyanine-resistant) occurs in all higher plants, in many fungi, and in some protozoa (Kirimura *et al.*, 1999; Johnson *et al.*, 2003). Such a pathway uses electrons from the ubiquinol pool to reduce oxygen to water, bypassing the complex III and the cytochrome oxidase complex, two sites of energy conservation in the main respiratory chain. An AOX, sensitive to SHAM and resistant to cyanide, is responsible for this alternative pathway (McIntosh, 1994). The cyanide-resistant respiration has been previously described in *C. albicans* and *C. parapsilosis* (Helmerhorst *et al.*, 2002; Chamilos *et al.*, 2006; Yan *et al.*, 2009). In this study, we described for the first time the presence of an ARP mediated by an AOX in *C. krusei*.

The elucidation of the oxidative stress responses in yeast has considerable clinical interest, as it is involved in invasion and colonization of host tissues by yeast pathogens as well as during the defensive mechanisms triggered by phagocytes. All aerobic organisms inevitably generate a range of ROS, including superoxide anion, hydrogen peroxide, and hydroxyl radical during oxygen metabolism. If not quickly and effectively eliminated from the cells, ROS will trigger a large number of oxidative reactions in cellular systems that possibly lead to cell death (Raha & Robinson, 2000). In the course of an *in vivo* infection, the formation of ROS and other oxidants radicals by phagocytes plays a crucial role in the intracellular destruction of the pathogen (Murphy, 1991). ROS attack almost all essential cell components, including DNA, proteins, and lipids (Moradas-Ferreira & Costa, 2000). In a recent study, *C. krusei* appears to be resistant to ROS and to possess a potent antioxidant system enabling deep systemic infections (Abegg *et al.*, 2010). We decided to evaluate the difference regarding oxidative stress response before and after the blockade of the ARP. It has been previously described that in *C. albicans*, fluconazole is able to induce the production and accumulation of ROS (Kobayashi *et al.*, 2002). In our study, we showed that fluconazole induced a low percentage of ROS formation by *C. krusei* cells. These results may suggest that the fungistatic mechanism of this azole is not based upon ROS formation. However, when the AOX was inhibited by SHAM, an increase in the intracellular ROS levels was evident. Attending to these facts, we can conclude that AOX activity allows the yeast cells to reduce ROS accumulation when challenged by antifungals like fluconazole, leading to drug tolerance, like in *C. albicans* (Yan *et al.*, 2009). According to several authors, the AOX has a metabolic and antioxidant role, and its presence may be considered a potential virulence attribute of pathogenic fungi (Vanlerberghe *et al.*, 2002; Missall *et al.*, 2004; Yan *et al.*, 2009). The importance of AOX activity upon resistance to oxidative stress was evident when the oxidative stress inductors, $H_2O_2$, menadione, and plumbagin, were assayed. After treatment with such compounds, ROS accumulation was low. However, the scenario changed significantly when the AOX activity was blocked by

SHAM. To confirm our hypothesis that the presence of an ARP could protect *C. krusei* from oxidative stress, we assessed ROS accumulation in the presence of fluconazole and the other oxidative stress inductors by the negative control strain, *S. cerevisiae* (AOX-). The results obtained regarding ROS accumulation, in the presence or absence of SHAM, were not significantly different. When testing *C. albicans* AOX mutant strains, Yan and coworkers also obtained no significant differences in the amount of ROS generation (Yan *et al.*, 2009). Regarding the chronological life span assays, we could conclude that the decreased viability of *C. krusei* strains in the presence of menadione and with the AOX blocked is associated with an increased level of cell ROS.

Our results showed clearly that the inhibition of the expression of the AOX was associated with intracellular ROS accumulation, revealing the effect of the ARP on oxidative damage. Although fluconazole resistance was unrelated to the presence of the ARP, we can consider that it confers antifungal tolerance, which may give yeast cells enough time to develop long-term genetically stable resistance mechanisms (Cannon *et al.*, 2009).

The ARP is a potential target that should be taken into account when considering the development of new therapeutic strategies in the case of *C. krusei* infections. Attending to its selective effect, SHAM should be used in combination with azoles, to reduce resistance because of oxidative stress and consequently the virulence of *C. krusei*.

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