ORIGINAL ARTICLE

Effect of phenotypic switching on the susceptibility of *Candida krusei* towards amphotericin B, nystatin and *Piper betle* aqueous extract

Mohd Hafiz Arzmi[1*], Fathilah Abdul Razak[2], Wan Himratul Aznita Wan Harun[2], Wan Nur Fatihah Wan Mohd Kamaluddin[3], Nurul Alia Risma Rismayuddin[3]

[1]Kulliyyah of Dentistry, International Islamic University Malaysia, Kuantan, Pahang, Malaysia
[2]Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia
[3]Kulliyyah of Nursing, International Islamic University Malaysia, Kuantan, Pahang, Malaysia

Abstract

Candida krusei (*C. krusei*) is associated with oral candidiasis, particularly in immune-compromised patients. The objective of the study was to determine the effect of phenotypic switching to the susceptibility of *C. krusei* towards amphotericin B, nystatin and aqueous extract of *Piper betle* (*P. betle*). To induce phenotypic switching, *C. krusei* was inoculated in yeast extract peptone dextrose (YEPD) broth supplemented with 5 mg mL^{-1} phloxine B and incubated for five hours at 25 °C. Later, 100 µL of the suspension was inoculated on YEPD agar supplemented with 5 mg mL^{-1} phloxine B and incubated for five days at 25 °C. Disc diffusion and minimum inhibitory concentration (MIC) assays were conducted to determine the susceptibility of *C. krusei*. The results showed that all *C. krusei* switched generations were susceptible towards amphotericin B and nystatin with the 3rd and 4th generations significantly more susceptible than the un-switched, respectively ($P<0.05$). All *C. krusei* switched generations were also observed to be susceptible towards the aqueous extract of *P. betle*. The MIC of amphotericin B, nystatin and *P. betle* were determined at 10 µg mL^{-1}, 10 µg mL^{-1} and 12.5 mg mL^{-1}, respectively for all generations of *C. krusei*. In conclusion, the susceptibility of *C. krusei* was phenotypically switched generation dependent towards amphotericin B, nystatin, and *Piper betle* aqueous extract.

Keywords: phenotypic switching, disk diffusion test, microdilution assay, metabolic activity

*Corresponding Author
Email: hafizarzmi@iium.edu.my
Tel: +60132601445

Introduction

Candida krusei (*C. krusei*) is associated with oral diseases such as oral candidiasis which is common in immuno-compromised patients (Muadcheingka & Tantivitayakul, 2015; *Mushi et al.,* 2016). The prevalence of *C. krusei* in patients with oral cancer has been reported to be higher than *Candida glabrata* and *Candida tropicalis* (Gravina *et al.*, 2007; Hautala *et al.*, 2007). *C. krusei* is an opportunistic microorganism that is resistant to fluconazole (Furlaneto-Maia *et al.,* 2008). Fluconazole is an antifungal agent that is usually prescribed for the treatment of candidal infection. Furthermore, *C. krusei* is also susceptible towards flucytosine, voriconazole, caspofungin, anidulafungin, micafungin and echinocandins. However, the sensitivity of the yeast towards these

antifungals has been reported to decrease (Hakki *et al.,* 2006; Pfaller *et al.,* 2008). Phenotypic switching is one of the important virulent factors of *Candida* species that enables the yeast to adapt in a stress environment (Jones *et al.,* 1994; Vargas *et al.,* 2004; Kang *et al.,* 2016; Palková & Váchová, 2016). It is a phenomenon that usually occurs in the oral environment of immuno-compromised patients (Morschhäuser, 2016). This mechanism of adaptation has been reported to associate with the alteration of genes that involve in the switching process such as metallothionein (*MT-2*), *SIR2* and mating-type loci (*MTL1)*, which may have a role in the decrease of sensitivity of *Candida* spp. towards antifungal agents (Brockert *et al.,* 2003; Low *et al.,* 2008; Arzmi *et al.,* 2012; Soll, 2014).

Amphotericin B and nystatin are used in the treatment of oral candidiasis. Amphotericin B is an antifungal agent that is prescribed in the treatment of primary oral candidiasis. This antifungal agent is also used as an adjunct to parenteral therapy in secondary candidiasis with systemic and topical manifestations on oral mucosal surfaces (Samaranayake *et al.,* 2009). Nystatin is another antifungal agent that is obtained from *Streptomyces noursei*, which is commonly used in the treatment of superficial fungal infection caused by *Candida* spp. (Silva *et al.,* 2013; Nenoff *et al.,* 2016).

Piper betle (*P. betle*) is a plant that belongs to the family of *Piperaceae* (Datta *et al.,* 2011). *Candida* has been reported to be susceptible to *P. betle* aqueous extract. However, there has been no study conducted with regard to the susceptibility of phenotypically switched *C. krusei*. (Himratul-Aznita *et al.,* 2011). In addition, *P. betle* oil-based extract has been reported to possess antibacterial and anti-protozoan that are effective in the treatment of *Salmonella typhi*, *Escherichia coli* and *Staphylococcus aureus* infections (Datta *et al.,* 2011; Himratul-Aznita *et al.,* 2011).

The objective of this study was to determine the effect of phenotypic switching to the susceptibility of *C. krusei* towards amphotericin B, nystatin and *P. betle* extract. It is hypothesised that the susceptibility of *C. krusei* towards amphotericin B, nystatin and *P. betle* extract is phenotypically switched generation dependent.

Materials and methods

Growth of microorganisms

C. krusei (ATCC 14243; Sigma-Aldrich, USA) was revived in yeast extract peptone dextrose (YEPD) broth (BD, USA) and incubated overnight at 37 ºC. Following that, 100 µL of the suspension was inoculated onto YEPD agar (BD, USA) and incubated at 37 ºC for 24 h.

Preparation of phenotypically switched *C. krusei*

C. krusei from YEPD agar was sub-cultured in YEPD broth supplemented with 5 mg mL^{-1} of phloxine B (Sigma-Aldrich, USA) to create a stress growth environment and incubated for 5 h at 37 ºC (Arzmi *et al.,* 2012). Following incubation, *C. krusei* cell density was standardised to 10^6 cells mL^{-1} that was equivalent to the optical density of 0.144 at 550 nm wavelength (OD_{550nm} of 0.144; Figure 1). The suspension was serially diluted, and 100 µL of the inoculum was pipetted on YEPD agar supplemented with 5 mg mL^{-1} of phloxine B. The suspension was spread evenly on the agar using a sterile glass spreader. The plate was incubated at 25 °C for five days. The plate that had approximately 50 colonies forming unit

(CFU) was examined for switched *C. krusei*. Colonies displaying morphological variation from the un-switched *C. krusei* were selected to represent the first switched generation (Arzmi *et al.,* 2012). Three colonies from the first switched generation that possessed similar morphology were collected and sub-cultured on to another set of YEPD agar supplemented with phloxine B to produce the 2nd, 3rd and 4th switched generations (Figure 2).

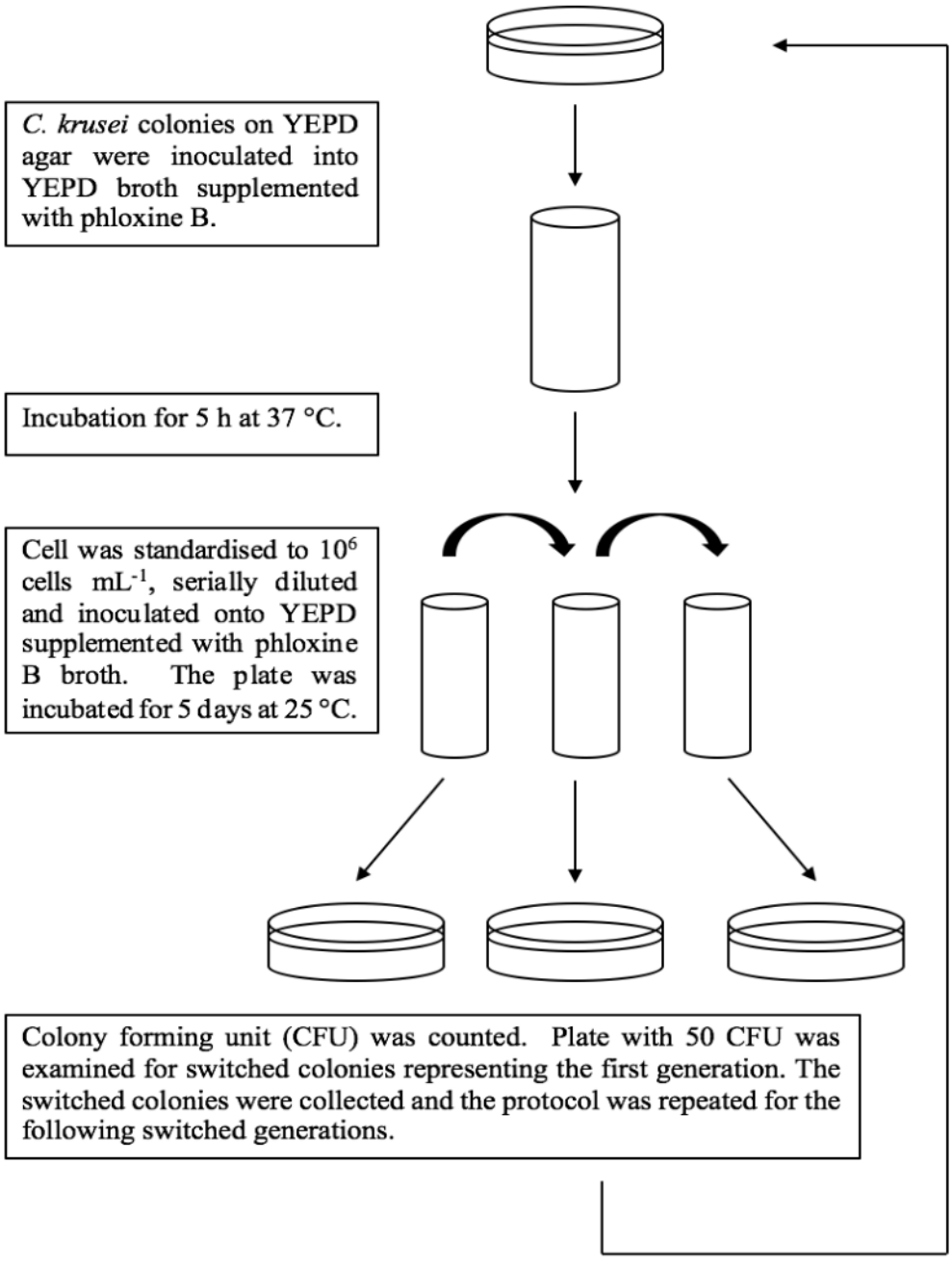

Figure 1. Illustration of protocol for phenotypic switching of *C. krusei*

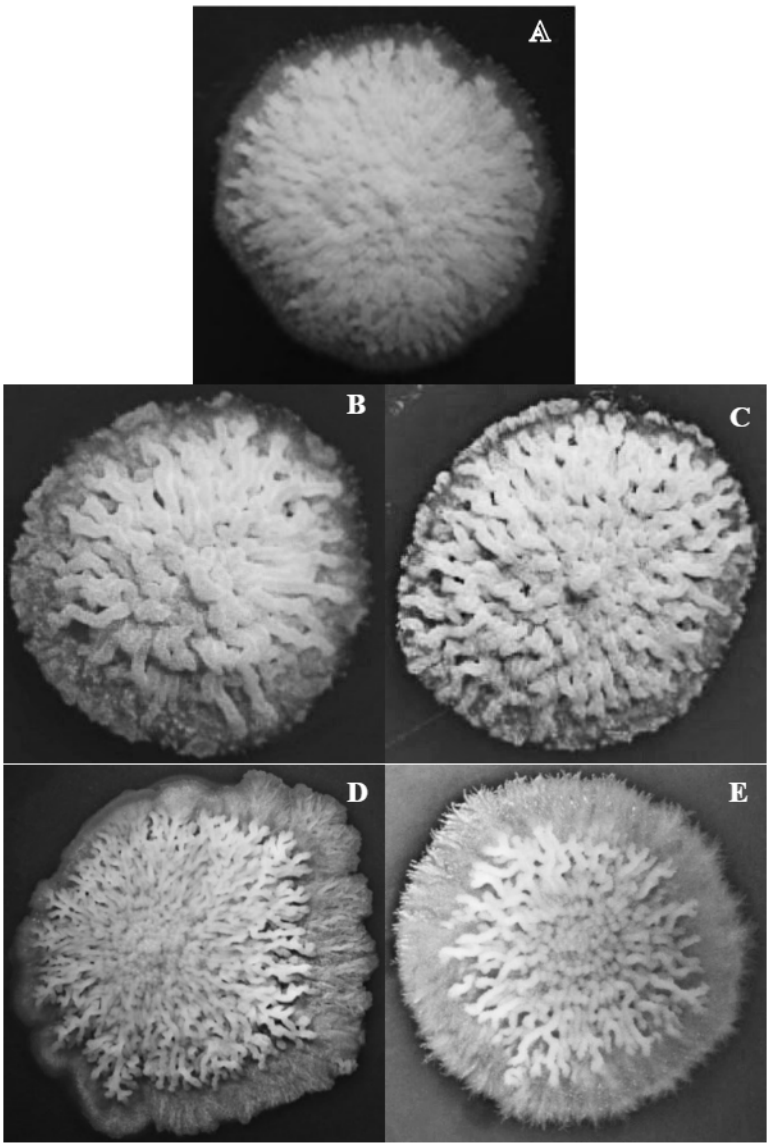

Figure 2. The colony of un-switched and switched *Candida krusei* at 10x magnification using a stereoscope. (A) Un-switched, (B) 1st switched generation, (C) 2nd switched generation, (D) 3rd switched generation, and (E) 4th switched generation (Arzmi *et al.*, 2012).

Determination of metabolic activity

The metabolic activity of un-unswitched and all switched generations of *C. krusei* was determined using BIOLOG YT MicroPlate (Hayward, CA). In brief, an overnight culture of *C. krusei* was suspended in 1 mL of sterile distilled water. Later, 100 µL of the suspension was inoculated in each well of YT MicroPlates following incubation at 25 °C for 24 h, 48 h and 72 h. The metabolic patterns were interpreted using BIOLOG's MicroLog 3 software and compared to the YT database.

Preparation of *P. betle* aqueous extract

P. betle leaves were freshly picked from Kampung Bukit Payong, Pokok Sena, Kedah, Malaysia. The leaves were cleaned with distilled water, and the total wet weight was recorded. The leaves were oven-dried at 60 °C for 48 h, and the total dry weight was recorded. Following that, 100 g of the leaves were cut into small pieces and put into a conical flask. The leaves were boiled in 2 L of distilled water until the volume was reduced to half (Himratul-Aznita *et al.*, 2011). The decoction was filtered into a

500 mL beaker, and the filtrate was re-boiled to a final volume of 100 mL. Finally, the concentrated extract was freeze-dried to produce *P. betle* water-based extract powder.

Disc diffusion test

The susceptibility of *C. krusei* towards amphotericin B, nystatin and *P. betle* aqueous extract was assessed by using disc diffusion test and microdilution method. Disc diffusion test was conducted by following the protocol of the National Committee for Clinical Laboratory Standards (CLSI, 2015a). To prepare *C. krusei* inoculum, the yeast colonies that were grown on YEPD agar supplemented with 5 mg mL^{-1} of phloxine B were suspended into 5 mL of 0.85% (v/v) of sterile saline. The optical density of the cell suspension was then standardised to an OD_{550nm} of 0.144 to give 10^6 cells mL^{-1} of *C. krusei*. Following that, 100 µL of the suspension was pipetted out and evenly swabbed on Mueller-Hinton (MH) agar (BD, USA). Amphotericin B (25 µg), nystatin (20 µg) and *P. betle* (20 mg) impregnated paper discs were aseptically placed onto the agar plate and incubated overnight at 37 °C. Finally, the diameter of the growth inhibition zone was measured. A similar protocol was conducted for all switched generations of *C. krusei* in triplicates.

Minimal inhibitory concentration assay

Minimum inhibitory concentration (MIC) was determined using the microdilution method as recommended by the National Committee for Clinical Laboratory standard (CLSI, 2015b). Initially, seven wells of a sterile 96-well plate (Nunc, USA) were labelled W1 to W7 horizontally (Table 1). Later, 100 µL of YEPD broth was added to W2 through W7, and 100 µL of the antifungal agent was added into W1 and W2. The plate was slowly agitated to mix the content. Following that, 100 µL of suspension in W2 was transferred to W3. Following thorough mixing, 100 µL of suspension in W3 was transferred to W4, and the procedure was continued through W6. After mixing, 100 µL from W6 was discarded. Finally, 100 µL of the yeast inoculum that was standardised to 10^6 cells mL^{-1} in YEPD broth was added to W1 through W7 and incubated at 37 °C for 24 h. W7 that received no antifungal treatment served as a negative control. The lowest concentration of antifungal agent that exhibited clear suspension was determined as the MIC. A similar protocol was repeated for all switched generations of *C. krusei* in triplicates.

Table 1. Illustration of minimum inhibitory concentration (MIC) determination.

Wells	W1	W2	W3	W4	W5	W6	W7
100 µL of YEPD broth		+	+	+	+	+	+
100 µL of antifungal agent	+	+	Serial dilutions were done until W6. Finally, 100 µL of suspension in W6 was discarded.				
100 µL of suspension	+	+	+	+	+	+	+

+ showed the presence of suspension in the well.

Statistical analysis

All data were statistically analysed using ANOVA post-hoc Tukey test on SPSS Statistic software version 25.0. The data were considered statistically significant if $P<0.05$.

Results

Metabolic activity of *C. krusei*

The un-switched an all switched generations of *C. krusei* were observed to ferment N-acetyl-D-glucosamine and α-D-glucose. However, only un-switched and 1st switched generation of *C. krusei* fermented γ-aminobutyric acid (GABA).

Susceptibility towards amphotericin B

Based on the disc diffusion test, the diameter of the inhibition zone of un-switched *C. krusei* when treated with amphotericin B was determined at 2.2 ± 0.1 cm (Table 2). The degree of susceptibility of the switched *C. krusei* towards amphotericin B increased gradually from the 1st, 2nd and 3rd generations with an inhibition zone of 2.3 ± 0.3 cm, 2.4 ± 0.1 cm and 2.6 ± 0.3 cm, respectively. The susceptibility was decreased in the 4th switched generation with an inhibition zone of 2.4 ± 0.1 cm. Finally, the MIC of amphotericin B towards un-switched and all switched generations of *C. krusei* were recorded at 10 µg mL^{-1}.

Susceptibility towards nystatin

The diameter of the inhibition zone for un-switched and the 1st switched generation of *C. krusei* were recorded at 2.4 ± 0.1 cm when treated with nystatin (Table 2). The susceptibility of *C. krusei* was decreased in the 2nd switched generation with an inhibition zone of 1.9 ± 0.2 cm, which was the least susceptible towards nystatin. In the 3rd and 4th switched generations, the susceptibility towards nystatin was observed to increase gradually with inhibition zones of 2.3 ± 0.1 cm and 2.6 ± 0.1 cm, respectively. The 4th switched generation was determined as the most susceptible among generations of *C. krusei*. The MIC of nystatin towards un-switched and all switched generations of *C. krusei* were determined at 10 µg mL^{-1}.

Susceptibility towards *Piper betle* aqueous extract

The un-switched *C. krusei* was susceptible towards *P. betle* aqueous extract with an inhibition zone of 2.2 ± 0.1 cm (Table 2). The degree of susceptibility of the 1st switched generation was increased compared to the un-switched *C. krusei*; however, no significant difference was observed ($P>0.05$). The inhibition zone of the 2nd switched geeration of *C. krusei* towards *P. betle* extract was recorded at 2.1 ± 0.1 cm, whereas the 3rd switched generation was determined at 2.1 ± 0.2 cm. The 4th switched generation was the least susceptible towards *P. betle* with an inhibition zone of 2.0 ± 0.2 cm. The MIC of *P. betle* aqueous extract was determined at 12.5 mg mL^{-1} for un-switched, and all switched generations of *C. krusei.*

Table 2. The effect of phenotypic switching on the susceptibility of *C. krusei* towards amphotericin B, nystatin and *Piper betle* extract.

Active ingredients	Amount of antifungal agent/extract	Growth generations				
		Un-switched	1st switched	2nd switched	3rd switched	4th switched
		Mean inhibition zone (cm)				
Amphotericin B	25 µg	2.2 (0.1)	2.3 (0.3)	2.4 (0.1)	2.6 (0.1)	2.4 (0.1)
Nystatin	20 µg	2.4 (0.1)	2.4 (0.1)	1.9 (0.2)	2.3 (0.1)	2.6 (0.1)
Piper betle	20 mg	2.2 (0.1)	2.3 (0.2)	2.1 (0.1)	2.1 (0.2)	2.0 (0.2)

Significantly more susceptible compared to un-switched ($P < 0.05$)
No significant different compared to un-switched ($P > 0.05$)
Significantly less susceptible compared to un-switched ($P < 0.05$)
The inhibition zones are the mean of three biological replicates. Standard deviation (SD) is given in the parenthesis.

Discussion

To our knowledge, this is the first study that emphasises on the effect of phenotypic switching to the susceptibility of *C. krusei* towards amphotericin B, nystatin and *P. betle* aqueous extract. The present study showed that the un-switched and all switched generations of *C. krusei* had different degrees of susceptibility towards amphotericin B with the 3rd generation exhibiting the least susceptible (Table 2). This finding supports the hypothesis of the study that the susceptibility of *C. krusei* towards amphotericin B is phenotypically switched generation dependent. Amphotericin B was reported to possess broad-spectrum fungicidal and fungistatic activities towards *Candida* spp. (Cuervo *et al.,* 2016). This antifungal has been shown to intercalate the membrane layer of *Candida* spp. to form channels that cause potassium ions to leak out, subsequently destroying the proton gradient (Kragelund *et al.,* 2016). Furthermore, it has been reported that amphotericin B can affect the sterol component of the yeast cell wall, which may have a role in the growth inhibition of *C. krusei* (Williams *et al.,* 2011).

The un-switched and all phenotypically switched generations of *C. krusei* were susceptible towards nystatin with the 2nd generation the least sensitive compared to other generations of *C. krusei*. This finding supports the hypothesis of the present study that the susceptibility of *C. krusei* towards nystatin is phenotypically switched generation dependent. The previous study has shown that nystatin can exhibit both fungistatic and fungicidal effects towards *Candida* spp. (Choudhary *et al.,* 2016). This antifungal agent can bind to the sterol component of the membrane, which subsequently affects the cell permeability of the yeast (Kragelund *et al.,* 2016). The previous study has shown that the interaction between nystatin and ergosterol component of the candidal cell membrane is responsible to the increase of cell porosity which leads to the loss of cytoplasmic substances (Williams *et al.,* 2011). It is postulated that the different degrees of *C. krusei* susceptibility towards nystatin is due to the phenotypic switching that may have altered the composition of ergosterol component in the cell membrane of the switched *C. krusei.* However, this warrants further study to ascertain this speculation.

Our study showed that *C. krusei* both un-switched and all switched generations were susceptible towards *P. betle* aqueous extract. However, no significant difference was observed between the switched generations (Table 1). This finding suggested that *P. betle* extract could be an effective antifungal agent towards *C. krusei*. *P. betle* extract is reported to contain hydroxychavicol which can destroy the cell membrane and reduce the cell viability of *Candida* spp. (Ali *et al.,* 2010). Furthermore, hydroxychavicol has also been reported as an effective antimicrobial agent towards *Microsporum gypseum* and *Streptococcus mutans* (Nalina & Rahim, 2007). Thus, further study to determine the specific and effective active compounds in *P. betle* aqueous extract using high-performance liquid chromatography (HPLC) would throw more light on its treatment of oral candidiasis.

The present study showed that only un-switched and 1st switched generation of *C. krusei* fermented GABA. GABA has been reported to be involved in the formation of succinate that is important in the energy production of a living cell (Bach *et al.,* 2009; Kregiel, 2012). The previous study has shown that the yeast cell wall stored a large portion of GABA indicating the potential role of this molecule in stress tolerance mechanism and as a nitrogen source (Coleman *et al.,* 2001; Bach *et al.,* 2009). This could explain the significant increase of susceptibility of the 3rd and 4th switched generations of *C. krusei* in the present study when treated with amphotericin B and nystatin, respectively (Table 2).

In conclusion, the present study confirmed that the susceptibility of *C. krusei* towards amphotericin B, nystatin and *P. betle* is phenotypically switched generation dependent that may have a role in the pathogenicity of the yeast in the oral cavity.

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