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Original Article

Comparison of the inhibition of *Candida spp.* biofilm formation by quorum-sensing molecules, farnesol and tyrosol with amphotericin B

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ABSTRACT

Background and Aims: Quorum sensing is a mechanism of cell-to-cell communication for controlling virulence and the biofilm formation of microorganisms. Due to this new treatment requiring approaches for biofilm-related infections, this study aims to examine the biofilm formation properties of both *Candida albicans* and non-albicans (NAC) strains and to compare the effects of the quorum-sensing molecules (QSMs) farnesol and tyrosol with the widely used antifungal agent amphotericin B with regard to biofilm attachment and biofilm formation.

Methods: Biofilm formation of 57 nonrepeat clinical isolates of *Candida spp*. (36 *C. albicans* and 21 NAC) was assessed through the crystal violet technique. Farnesol (300 µM), tyrosol (80 µM), and amphotericin B (4 µg/ml) were evaluated against biofilm attachment and biofilm formation (plates incubated 2, 4, 6 and 24 h).

Results: All isolates displayed biofilm-forming capabilities. *C. albicans* demonstrated mostly weak biofilm formation (42.2%), whereas the NAC species showed strong biofilm formation (52.38%). Depending on the stage at which they were added, farnesol and tyrosol significantly inhibited the biofilm formation of *C. albicans* and NAC species, especially at 6 h, which is the early stage of biofilm development. Unfortunately, QSM activity decreased at 24 h. In addition, amphotericin B showed a stronger inhibitory effect than the QSMs at all time points studied, with up to 60% inhibition being observed.

Conclusion: QSMs can significantly inhibit biofilm development in both *C. albicans* and NAC species depending on the stage when they are added, especially in the early stages of biofilm formation.

Keywords: *Candida spp*., biofilm, quorum sensing, farnesol, tyrosol, amphotericin B

INTRODUCTION

Recent years have seen a notable increase in the prevalence of human fungal infections, particularly *Candida* infections which include invasive candidiasis, oropharyngeal/oral candidiasis, and vulvovaginal candidiasis. The main contributing factors have been the Acquired Immune Deficiency Syndrome epidemic, an increasingly aging population, an increase in immunocompromised patient numbers, and also the increased use of indwelling medical devices (Silva et al., 2012). While *Candida albicans* is the most commonly isolated *Candida* species among humans (representing over 80% of all candidiasis cases in humans), the prevalence of infections due to non-albicans *Candida* (NAC) species such as *Candida tropicalis, Candida glabrata, Candida parapsilosis, Candida krusei*, and *Candida kefyr* over the last two decades has increased significantly (Chin, Lee, Rusliza, & Chong, 2016; Silva et al., 2012).

Although *Candida* species are present on the mucosal membranes and skin of normal individuals, they can cause infection under specific circumstances. The most important step in the *Candida* infection is the adhesion to host surfaces, as this is necessary for initial colonization and can lead to the organism's persistence. Adherence is considered critical for establishing disease. Additionally, *Candida* species have also have the ability to attach to the surfaces of medical equipment and develop biofilms (Malinovská, Čonková, & Váczi, 2023; Silva et al., 2012). Biofilms are organized communities of microorganisms that adhere to a surface or interface and are surrounded by a self-produced exopolymeric matrix. Microorganisms predominantly exist as biofilms in the natural environment or within human systems. Biofilms are estimated to be associated with 80% of microbial infections in humans and in the natural environment (Brackman & Coenye, 2015). Systemic mycoses, for which *Candida* is responsible in 90% of cases, are strongly as-

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sociated with biofilm formation. According to recent statistics, systemic mycoses unfortunately result in approximately 1.6 million human deaths each year (Malinovská et al., 2023; Nami, Aghebati-Maleki, Morovati, & Aghebati Maleki, 2019). In addition, the mortality rate for *Candida*-related bloodstream infections is 37.9%. Of these, 70.0% are due to biofilm-related infections (Atiencia-Carrera, Cabezas-Mera, Tejera, & Machado, 2022).

Quorum sensing (QS) is a mechanism of cell-to-cell communication for controlling virulence and biofilm formation and occurs through the continuous release and monitoring of hormone-like molecules known as autoinducers, or quorum-sensing molecules (QSMs; Albuquerque & Casadevall, 2012).

QS regulation was first discovered in bacteria in the 1960s and 1970s during studies of genetic competence in *Streptococcus pneumoniae* and bioluminescence in marine *Vibrio* species. Until farnesol was discovered as a QSM in *C. albicans*, QS in eukaryotic organisms had been unknown. Since then, studies have shown a large number of structurally distinct fungal QSMs, such as tyrosine (tyrosol), phenylalanine (phenylethanol), and tryptophan (tryptophol), that are responsible for a variety of properties in evolutionarily divergent fungi (Albuquerque & Casadevall, 2012; Tian, Ding, Ke, & Wang, 2021).

Although its concentration and production are highest in *C. albicans*, the first eukaryotic QSM (i.e., farnesol) is able to be secreted by eight *Candida* under different conditions: *C. albicans, C. tropicalis, C. parapsilosis, C. kefyr, C. krusei, C. glabrata, Candida dubliniensis* and *Candida guilliermondii* (Weber, Sohr, Schulz, Fleischhacker, & Ruhnke, 2008). Farnesol is involved in inhibiting hypha formation and in regulating various physiological processes, including filamentation, drug efflux, and apoptosis. Farnesol has also been shown to be able to reversibly inhibit biofilm formation without blocking the elongation of pre-existing hyphae (De Sordi & Mühlschlegel, 2009; Rodrigues & Černáková, 2020).

Tyrosol is the another major QSM secreted by *C. albicans* and is released into the culture medium continuously as the organism grows. Tyrosol reduces the duration of the lag phase of growth and stimulates filamentation and biofilm formation during the early steps of biofilm development (1-6 h). Therefore, its effects are particularly strong during the early and intermediate periods of biofilm formation (Albuquerque & Casadevall, 2012; Rodrigues & Černáková, 2020).

New treatment approaches are well known to be required for biofilm-associated infections, which are notoriously difficult to treat. Additionally, eradicating mature biofilms is a challenging task. This study investigates the biofilm formation properties of both *C. albicans* and NAC strains and compares the effects of two important QSMs (i.e., farnesol and tyrosol) on the biofilm formation of these strains with that of amphotericin B.

MATERIALS AND METHODS

Fungal isolates

A total of 57 *Candida* spp. (nonrepeat), including 36 *C. albicans* and 21 NAC species (6 *C. glabrata*, 6 *C. tropicalis*, 5 *C. parapsilosis*, 2 *C. famata*, 1 *C. kefyr*, and 1 *C. krusei*) contained in various clinical samples from blood, urine, vagina, ear, esophagus, sputum, endotracheal aspirate, and wounds were submitted to the Synevo Laboratories Ankara Central Laboratory in Türkiye (2021-2022). The Vitek 2 (BioMerieux, France) and CHROMagar *Candida* (BioMerieux, France) systems were used to identify the strains. Each isolate was grown on Sabouraud dextrose agar (SDA, Merck) to ensure viability before analysis.

QSMs and Amphotericin B

The QSMs of farnesol and tyrosol were used in respective 300 and 80 μM concentrations in the experiments and were purchased from Sigma-Aldrich. Amphotericin B (4 μg/ml) was kindly provided by the manufacturer, and stock solutions were prepared from dry powders in water and stored frozen at -80°C; the frozen solutions were used within 6 months. The antimicrobial concentrations that were used are based in part on those used in previous studies and the closest toxic concentrations (Hacioglu, Haciosmanoglu, Birteksoz-Tan, Bozkurt-Guzel, & Savage, 2019b; Katragkou et al., 2015; Shanmughapriya, Sornakumari, Lency, Kavitha, & Natarajaseenivasan, 2014).

Biofilm formation

Biofilms were formed in microtiter plate wells using clinical isolates of *C. albicans* and NAC species according to the protocol of Ramage, Vande, Wickes, & Lopez-Ribot (2001). Overnight cultures of isolates from 24-h growth on yeast extract-peptone-dextrose agar (Sigma-Aldrich) were inoculated into yeast extract-peptone-dextrose broth (YPDB, Sigma-Aldrich) and incubated overnight at 37°C on an orbital shaker. Cultures were centrifuged (3000 rpm for 5-10 min), rinsed twice with sterile phosphate-buffered saline (PBS) and resuspended in YPDB at a cell density equivalent to 1×10^6 cells/mL. For biofilm formation, 100 μL of the standardized cell suspension was carefully pipetted into the selected wells of sterile flat-bottomed 96-well polystyrene tissue culture plates (Greiner Bio-One, Germany) and incubated at 37°C for 24 h. After incubation, the waste medium was gently aspirated, and non-adherent cells were removed by washing the biofilms three times with sterile PBS.

Quantification of biofilm formation

Biofilm formation of the clinical isolates of *C. albicans* and NAC species was assessed using the crystal violet technique as described by Djordjevic, Wiedmann, & McLandsborough

(2002) and Hacioglu, Tan, Dosler, Inan, & Otuk (2018). In brief, after the formation of the biofilm, each well was washed twice with 200 ml of PBS and air dried. Each washed well was then stained for 45 min with 100 ml of 0.4% aqueous crystal violet solution. These wells were then washed four times again with sterile PBS and instantly distained with 200 ml of 95% ethanol. After 45 min 100 ml of the distaining solution was transferred to a new well. The amount of crystal violet staining in the distaining solution was measured using a microtiter plate reader (Bio-Rad Novapath, California, USA) at 595 nm. In order to reduce background interference, the negative controls' absorbance values (containing no cells) were removed from the values of the test wells. Biofilm formation was measured in triplicate for each isolate using the wild-type strain of *C. albicans* (SC 5314) as the strong biofilm producer. Weak, moderate, and strong biofilm formation were defined as follows:

OD (optical density) (isolate) ≤ OD (negative control) = negative biofilm formation;

OD (negative control) ≤ OD (isolate) ≤ 2xOD (negative control) = weak biofilm formation;

2xOD (negative control) ≤ OD (isolate) ≤ 4xOD (negative control) = moderate biofilm formation;

4xOD (negative control) ≤ OD (isolate) = strong biofilm formation (Oyardi, Hacioglu, Yilmaz, Inan, & Bırteksoz Tan, 2023)

Inhibition of biofilm attachment and biofilm formation

Overnight cultures of isolates were prepared in YPDB so as to result in a cell density equivalent to 1×10^6 cells/mL. The clinical isolates of the *C. albicans* and NAC species were added to each well of tissue culture microtitration plates (96 wells) containing the QSMs and amphotericin B. Positive controls with no antimicrobials and negative controls with no cells were also added. Plates were incubated at 37°C for 2, 4, 6, and 24 h. After incubation, the wells were washed twice with PBS, and the optical density (OD) was read spectrophotometrically at 450 nm using a microplate reader (Bio-Rad Novapath, California, USA; Bozkurt-Guzel, Hacioglu, & Savage, 2018).

RESULTS

Biofilm formation assay

All isolates displayed biofilm-forming capabilities. In particular, *C. albicans* demonstrated mostly weak biofilm formation (42.2%), whereas the NAC species showed strong biofilm formation (52.38%). The results are shown in Table 1.

Effects of QSMs and amphotericin B on *C. albicans* biofilm attachment and biofilm formation

Although the inhibition of adhesion and biofilm formation rates was time-dependent, the range of percentage inhibition rates (%) for farnesol and tyrosol was found to be between 3.18 and 31.13 for farnesol and between -5.31 and 12.65 for tyrosol. Both QSMs showed the highest inhibitory effect at 6 h, while their activity decreased at 24 h. Amphotericin B showed a stronger inhibitory effect than the QSMs for all time points studied (Figure 1a).

Effects of QSMs and amphotericin B on NAC biofilm attachment and biofilm formation

The time-dependent inhibition rates (%) of farnesol and tyrosol respectively ranged between -3.47 and 20.78 and between -2.47 and 9.59. Farnesol was more effective than tyrosol except at time zero. The most potent antimicrobial agent with up to 60% inhibition rates was found to be amphotericin B (Figure 1b).

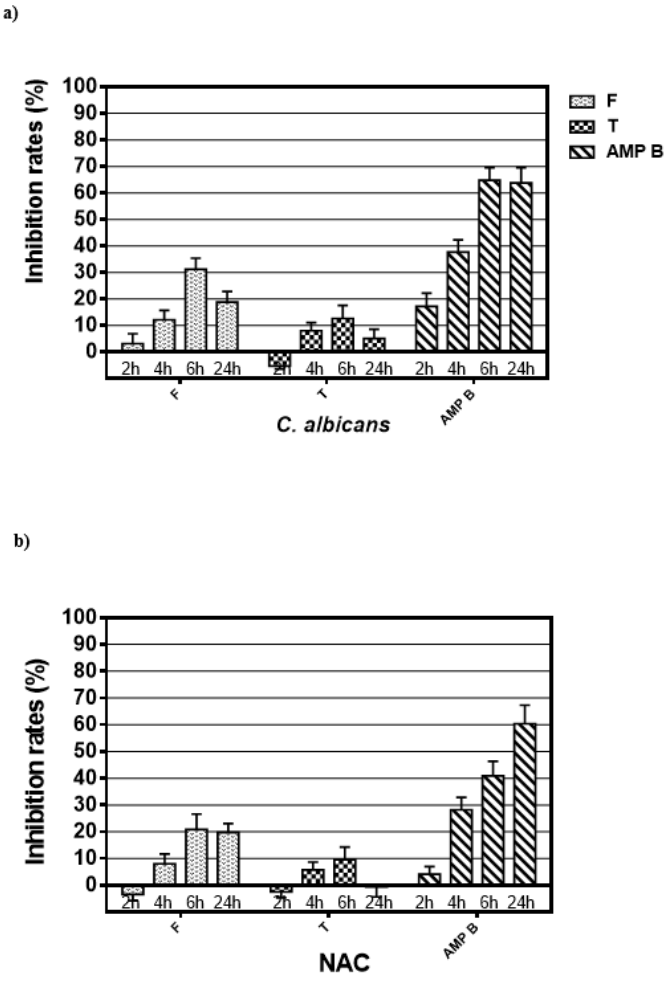

Figure 1. Effects of QSMs and amphotericin B on *Candida spp*. biofilm attachment and biofilm formation. a) *C. albicans* b) NAC. Inoculums were incubated for 2, 4, 6 and 24 hat 37°C. Four wells were used for each agent, and each experiment was performed with at least two independent tests. The error bars indicate the standard deviations.
F = farnesol; T = tyrosol; AMP B = amphotericin B

Table 1. Biofilm formation rates of isolates

	Biofilm formation capabilities (%)		
Strains	**Strong**	**Moderate**	**Weak**
***C. albicans* (n=36)**	22.2 (n=8)	30.55 (n=11)	42.2 (n=17)
NAC (n=21)	52.38 (n=11)	28.57 (n=6)	19.04 (n=4)
C. glabrata (n=6)	50 (n=3)	33.3 (n=2)	16.6 (n=1)
C. tropicalis (n=6)	50 (n=3)	-	50 (n=3)
C. parapsilosis (n=5)	60 (n=3)	40 (n=2)	-
C. famata (n=2)	50 (n=1)	50 (n=1)	-
C. kefyr (n=1)	100 (n=1)	-	-
C. krusei (n=1)	-	100 (n=1)	-

DISCUSSION

Candida is an opportunistic yeast genus and is the fourth most common cause of hospital-acquired bloodstream infections. It is responsible for 90% of systemic mycoses, with around 70% of candidemia being closely related to biofilm formation. These infections cause approximately 1.6 million human deaths each year. *Candida* biofilms can be seen on both living surfaces such as mucous membranes, organs, and blood vessels, as well as on non-living surfaces such as medical devices (e.g., medical catheters contact lenses, cardiovascular implants, pacemakers, shunts; Atiencia-Carrera et al., 2022; Malinovská et al., 2023). Biofilms are well known for being able to lead to significant resistance to antifungal agents. Therefore, a pressing need exists for novel agents that target biofilm and that operate through unique mechanisms such as QSMs. For this purpose, the aim of the present study has been to investigate the biofilm formation properties of both *C. albicans* and NAC species and to compare the effects of two important QSMs (i.e., farnesol and tyrosol) against amphotericin B regarding biofilm formation.

The study's results demonstrate all of the isolates to have produced biofilms; however, the NAC strains showed stronger biofilm formation rates (52.38%) than the *C. albicans* strains (22.2%). Previous studies have shown *Candida* isolates from different clinical materials to be able to form biofilms at different rates. One study observed biofilm formation in 92% of *C. albicans* isolates (n=77), and 100% of NAC species (n=16) that had been isolated from vulvovaginal candidiasis patients showing the ability to form biofilms (Hacioglu, Guzel, Savage, & Tan, 2019a). Atiencia-Carrera et al. (2022) investigated the rate, type, and antifungal resistance of *Candida* biofilms among hospitalized patients for the years 1995 and 2020. According to their results, the low, intermediate, and high biofilm rates were 36.2%, 18.9%, and 35%, respectively, with *C. tropicalis* being the predominant species in high biofilm formation (67.5%) and *C. krusei* and *C. glabrata* being the second and third most common strong biofilm producers among the *Candida* species.

In line with emerging areas of research focusing on the prevention of microorganisms' adherence and biofilm development, the present study has investigated farnesol and tyrosol's and amphotericin B's ability to inhibit biofilm attachment and biofilm formation in the clinical isolates of *C. albicans* and NAC species.

Although biofilm formation is a process present in all *Candida* species, which is able to form a biofilm in 38-72 h, this formation is influenced by several factors and varies significantly from species to species (Malinovská et al., 2023). *C. albicans* biofilm formation has been characterized by three stages of development: 1) adhesion of the yeast cells (early stage: 0–11 h), 2) yeast cell differentiation into hyphal cells (intermediate stage: 12–30 h), and 3) matrix increase (maturation stage: 31–72 h; Atriwal et al., 2021; Tobudic, Kratzer, Lassnigg, & Boucherit, 2012). According to the current study's results regarding *C. albicans*, inhibition rates for farnesol and tyrosol were respectively found to be 31.13% and 12.65% at 6 h, which is the early stage of biofilm development. Unfortunately, these QSMs' activity decreased at 24 h.

C. albicans biofilms exhibit a heterogeneous structure comprised of cells, hyphae, and pseudohyphae enclosed by an extracellular matrix (ECM) material. The ECM provides a structural support for adhesion between biofilm cells and biotic or abiotic surfaces. It also acts as a barrier between the biofilm cells and the surrounding environment. Among the NAC species, *C. glabrata* is not polymorphic, does not form true hyphae or pseudohyphae, and has a biofilm consisting of yeast cells in a multilayered organization (i.e., cell clusters). The biofilms of *C. tropicalis* and *C. parapsilosis* have a biofilm structure consisting of yeasts and pseudohyphae surrounded by a minimal ECM with low carbohydrate and protein content (Cavalheiro & Teixeira, 2018; Malinovská et al., 2023). The present study

found the QSMs of farnesol and tyrosol to inhibit biofilm attachment of NAC species at 20.78% and 9.59%, respectively at 6 h.

The QSMs of farnesol and tyrosol work together in the fungal life cycle to aid fungal adhesion, proliferation, filamentation, and dispersal. Farnesol prevents hyphae formation and inhibits biofilm formation when added at beginning of biofilm formation, whereas tyrosol stimulates hyphal formation, particularly in the early and intermediate stages of biofilm formation (Cordeiro et al., 2015; Fourie & Pohl, 2019). The current study's results have also confirmed farnesol to be able to significantly inhibit biofilm formation when added at the early stages of biofilm formation, whereas tyrosol promoted biofilm growth. The biofilm inhibition rates for tyrosol at 2 h were -5.31% and -2.47% respectively for both the *C. albicans* and NAC species. However, tyrosol showed biofilm inhibition at 4 and 6 h for both *C. albicans* and NAC species. According to Sebaa, Boucherit-Otmani, & Courtois (2019), farnesol at 3 mM and tyrosol at 20 mM showed stronger inhibition when added at the beginning of biofilm formation (>50% and >80% inhibition, respectively) than when added to preformed biofilms (<10% and <40% inhibition, respectively). Other studies have also shown farnesol and tyrosol to be able to be effective against *Candida* biofilms, depending on the concentration and stage when they are added (Alem, Oteef, Flowers, & Douglas, 2006; Yapıcı, Gürsu, & Dağ, 2021).

The polyene antifungal agent amphotericin B is widely used in the treatment of invasive fungal infections, such as candidiasis and biofilm infections and is considered to be the most reliable and widest spectrum treatment against these types of infections (Hamill, 2013; Touil, Boucherit-Otmani, & Boucherit, 2018). The current study found amphotericin B to show a stronger inhibitory effect than the QSMs at all time points studied, with up to 60% inhibition. Another study investigated the inhibition of biofilm formation for 23 clinical *Candida* isolates (10 *C. krusei* and 13 *C. tropicalis*) using amphotericin B, tyrosol, and the combination of the two. The inhibition of biofilm by amphotericin B or tyrosol was shown to be concentration dependent, with a 50% reduction at 4 µg/ml and 80 µM, respectively (Shanmughapriya et al., 2014).

In conclusion, the QSMs of farnesol and tyrosol work together to regulate fungal morphology, especially with regard to biofilms. The study's results show these QSMs to be able to significantly inhibit biofilm formation in both *C. albicans* and NAC species, depending on the stage when they are added and especially during the early stages of biofilm formation. The antibiofilm activity of QSMs may be of interest for developing new antifungal strategies due to the clinical importance of *Candida* biofilm infections.

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