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Candida tropicalis Biofilms: Biomass, Metabolic Activity and Secreted Aspartyl Proteinase Production

Melyssa Negri · Sónia Silva ·
Isis Regina Grenier Capoci · Joana Azeredo ·
Mariana Henriques

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Abstract According to epidemiological data, *Candida tropicalis* has been related to urinary tract infections and haematological malignancy. Several virulence factors seem to be responsible for *C. tropicalis* infections, for example: their ability to adhere and to form biofilms onto different indwelling medical devices; their capacity to adhere, invade and damage host human tissues due to enzymes production such as proteinases. The main aim of this work was to study the behaviour of *C. tropicalis* biofilms of different ages (24–120 h) formed in artificial urine (AU) and their ability to express aspartyl proteinase (*SAPT*) genes. The reference strain *C. tropicalis* ATCC 750 and two *C. tropicalis* isolates from urine were used. Biofilms were evaluated in terms of culturable cells by colony-forming units enumeration; total biofilm biomass was evaluated using the crystal violet staining method; metabolic activity was evaluated by XTT assay; and *SAPT* gene expression was determined by real-time PCR. All strains of *C. tropicalis* were able to form biofilms in AU, although with differences between strains. *Candida tropicalis* biofilms showed a decrease in terms of the number of culturable cells from 48 to 72 h. Generally, *SAPT3* was highly expressed. *C. tropicalis* strains assayed were able to form biofilms in the presence of AU although in a strain- and time-dependent way, and *SAPT* genes are expressed during *C. tropicalis* biofilm formation.

Keywords *Candida tropicalis* · Biofilm · Artificial urine · *SAPT* gene

M. Negri · S. Silva (✉) · J. Azeredo · M. Henriques
CEB- Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
e-mail: soniasilva@deb.uminho.pt

M. Negri · I. R. G. Capoci
Teaching and Research in Clinical Analysis Laboratory, Division of Medical Mycology, Universidade Estadual de Maringá, Av. Colombo, 5790, Maringá, Paraná 87020-900, Brazil

Introduction

Candida tropicalis ranks between the second and third non-*Candida albicans Candida* species (NCAC) most frequently isolated from patients with *Candida* infections [1–5]. According to epidemiological data, *C. tropicalis* has been related to urinary tract infections and haematological malignancy [3, 4, 6, 7]. Furthermore, the most important causes of *C. tropicalis* candiduria are associated with antibiotic therapy and indwelling catheterization [8–10]. Several virulence factors seem to be responsible for *C. tropicalis* infections, for example: their ability to adhere and to form biofilms onto different indwelling medical

devices; their capacity to adhere, invade and damage host human tissues due to enzymes production such as proteinases [9, 11–16]. Formation of *C. tropicalis* biofilms has important clinical repercussions because of their increased resistance to antifungal therapy and the ability of cells within biofilms to withstand host immune defences [17, 18]. This is important because, according to epidemiological data, *C. tropicalis* infection is strongly connected with the presence of biofilms in urinary catheters [12, 19–23]. In the last decade, early events associated with *C. tropicalis* biofilm formation have received considerable attention [11, 12, 14, 16, 24]. However, very little is known about the behaviour and presence of enzymes, such as proteinases, in *C. tropicalis* biofilm formation. Furthermore, recent studies with *C. albicans* demonstrated that biofilm cells display a distinct phenotype, which is associated with an increased virulence, such as the ability to produce hydrolytic enzymes [25]. Thus, the aim of this study was to investigate the behaviour of *C. tropicalis* biofilms formed in the presence of artificial urine and their ability to express aspartyl proteinase (*SAPT*) genes.

Materials and Methods

Candida tropicalis and Growth Conditions

Three strains of *C. tropicalis* were used in this study: one reference strain from the American Type Culture Collection (ATCC 750) and two clinical isolates (U69 and U75) obtained from patients with candiduria admitted to the intensive care unit and belonging to the archive collection of the University Hospital in Maringá, Paraná, Brazil. The yeasts were identified by three methods: the MicroScan rapid yeast identification panel (Dade Behring Inc, CA, USA), the classical biochemical method and molecular identification [15]. The strains were kept frozen at −80 °C in Sabouraud dextrose broth (SDB; Liofilchem, Italy) containing 5 % (v v^{-1}) glycerol. For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) for 48 h at 37 °C. Yeast cells were then inoculated in Sabouraud dextrose broth (SDB; Merck) and incubated for 18 h at 37 °C under agitation in an orbital shaker (120 rev/min). After incubation, yeast cells were harvested by centrifugation at 8000×*g* for 5 min at 4 °C and washed twice with phosphate buffer solution (PBS; pH 7.5; 0.01 mol l^{-1}). The remaining pellets were suspended in artificial urine (AU), and the cellular density adjusted to 1×10^7 yeasts mL^{-1}, using a Neubauer chamber. Artificial urine (pH 5.8) was prepared according to Silva et al. [11].

Candida tropicalis Biofilms Formation

Biofilms, with different ages of maturation (24, 48, 72, 96 and 120 h), were formed on silicone coupons (1×1 cm^{-2}) according to Silva et al. [11]. The coupons were placed in 24-well microtiter plates (Orange Scientific, Braine-l′Alleud, Belgium), and 1 mL of standardized *C. tropicalis* suspension (1×10^7 yeasts mL^{-1} in AU) was added to each well. The microtiter plates were incubated for 24–120 h at 37 °C in an orbital shaker (120 rev/min). Every 24 h, an aliquot of 500 μL of AU was removed and an equal volume of fresh AU added to each well. The silicone coupons used as controls were similarly treated but in the absence of *C. tropicalis*. After the defined times of incubation, the medium was aspirated and non-adherent *C. tropicalis* cells were removed by washing the silicone coupons with PBS.

Candida tropicalis Biofilm Characterization

Biofilms, recovered at each time point, were evaluated in terms of: (1) number of culturable yeasts by colony-forming units (CFU) enumeration; (2) total biofilm biomass using the crystal violet (CV) staining method; (3) metabolic activity by 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. The results of CV and XTT assays were classified according to the cut-offs proposed by Marcos-Zambrano et al. [26]. The cut-offs used were as follows: low (<0.44), moderate (0.44–1.17) and high (>1.17) biofilm forming; and low (<0.097), moderate (0.097–0.2) and high (>0.2) metabolic activity. All experiments were repeated on three occasions with individual samples evaluated in triplicate.

Number of Culturable Yeasts

The number of culturable yeasts was determined by CFU enumeration, according to Silva et al. [14] with some modifications. Briefly, 1 mL of PBS was added

to the silicone coupons (containing the washed biofilms), and the biofilms were removed with a cell scraper (Orange Scientific, Belgium). The coupons, immersed in PBS, were sonicated (Ultrasonic Processor; Cole-Parmer) for 45 s at 30 W (parameters optimized to avoid cell lysis). The suspensions obtained were vortexed vigorously for 5 min, and then serial decimal dilutions (in PBS) were plated onto SDA. Agar plates were incubated for 24 h at 37 °C, followed by CFU enumeration, and the results were recorded as CFU per unit area of coupon (CFU cm^{-2}).

Biofilm Biomass Quantification by Crystal Violet Staining

CV staining was used to assess total biomass quantification (yeast, pseudohyphae, hyphae and matrix components). Thus, at the defined time points of incubation, the biofilms were stained in accordance with Silva et al. [14]. The final absorbance values were standardized according to the area of silicone coupons (absorbance cm^{-2}).

In Situ Biofilm Metabolic Activity

After biofilm formation (as described previously), the reduction assay of the tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT; Sigma-Aldrich, USA) [14] was used to determine the in situ biofilm mitochondrial activity of *C. tropicalis* cells in the biofilms. The absorbance values were standardized per unit area of well (absorbance cm^{-2}).

Analysis of *SAP* Gene Expression

SAP gene expression was evaluated for planktonic and biofilm cells. For planktonic cells, a suspension of *C. tropicalis*, adjusted to 1×10^7 yeasts mL^{-1} in AU, was incubated for 24 h at 37 °C under agitation in an orbital shaker (120 rev/min). Finally, the yeast cells were harvested by centrifugation at $8000\times g$ for 5 min at 4 °C, and the pelletized cells were suspended in 500 µL of lysis buffer (Invitrogen, USA). After biofilm formation on silicone, as described above, the biofilms were scraped from the coupons into 500 µL of lysis buffer.

RNA Extraction

Candida tropicalis samples were transferred to screw-cap tubes (Bioplastics, NL), and glass beads (0.5 mm diameter, approximately 500 µL) were added before the tubes were homogenized twice for 30 s, using a Mini-BeadBeater-8 (Stratech Scientific, Soham, UK). After yeast cells disruption, the PureLink™ RNA Mini Kit (Invitrogen) was used for total RNA extraction according to the manufacturer's recommended protocol. To avoid potential DNA contamination, the samples were treated with RNase-free DNase I (Invitrogen).

Primers

The primers used for real-time PCR (RT-PCR) were described in Silva et al. [27] and Negri et al. [7]. The pairs of primers were as follows: 5′-GGAAGATCT GATGTGCCAACTACATTGA-3′ and 5′CGTGCGG CCGCTCTACAAAGCCGAGATGTCT-3′ for *SAP1*, 5′-TTCTTCTAGTGGTACCTGGGTCAAAG-3′ and 5′-CATAGATCTCTAAACAATAGTGACATTAG A-3′ for *SAP2*, 5′-ACTTGGATTTCCAGCGAAGA-3′ and 5′-AGCCCTTCCAATGCCTAAAT-3′ for *SAP3*, 5′-GTACTCGAGCTCCTACAACTTCACC TCCT-3′ and 5′-CATGGATCCCTATGTAAGTG GAAGTATGTT-3′ for *SAP4*, 5′-GACCGAAGCTC CAATGAATC-3′ and 5′-AATTGGGACAACGT GGGTAA-3′ for *ACT1*. The efficiency of primers *SAPT1-4* and actin 1 (*ACT1*) as a reference housekeeping gene in concentration of 2 µM was evaluated using melting curve, percentage of efficiency and dilution with best standard curve generated by StepOne™ software version 2.3 in titration experiments using complementary DNA (cDNA) of *C. tropicalis* ATCC 750 both planktonic and biofilm cells in serial log10 dilutions.

Synthesis of cDNA

To synthesize the cDNA, the iScript™ cDNA Synthesis Kit (Bio-Rad, USA) was used according to the manufacturer's instructions. For each sample, 10 µL of extracted RNA was used.

Real-Time PCR

Real-time PCR (CF X96™ Real-Time PCR System, Bio-Rad, USA) was used to determine the relative levels of *SAPT1-4* mRNA transcripts with (*ACT1*) as a reference housekeeping gene according to Silva et al. [27] and Negri et al. [7]. Each reaction mixture consisted of: working concentration of SsoFast™ EvaGreen® Supermix (Bio-Rad, USA), 300 nmol forward and reverse primer and 1 µL of cDNA, in a final reaction volume of 20 µL. Negative controls (water) were included in each run. The relative quantification of *SAPT1-4* gene expression was performed by the ΔC_T method [7, 27]. Each reaction was performed in triplicate, and mean values of relative expression were analysed for each *SAP* gene.

Statistical Analysis

The results obtained were analysed using the SPSS 18 (Statistical Package for the Social Sciences) program. One-way ANOVA with the Bonferroni test was used in these tests. All tests were performed with a confidence level of 95 %. All the experiments were performed in triplicate and in three independent assays.

Results

Candida tropicalis Biofilm Characterization

Figure 1 presents the evaluation of biofilm formation by the different *C. tropicalis* strains along time. It can be verified that all strains were able to form biofilms in silicone and in the presence of artificial urine (AU) according to the results of number of cultivable yeasts, total biomass and in situ mitochondrial biofilm metabolic activity. The number of culturable yeasts (Fig. 1a) from U75 and ATCC 750 biofilms was similar in all time points assayed. However, the clinical isolate U69 showed significantly less ($p = 0.01$) culturable yeasts (1.60×10^5 CFU cm^{-2}) from 24-h-old biofilms and higher number of culturable yeasts (1.08×10^7 CFU cm^{-2}) from 48-h biofilms, than the other two strains. In general, *C. tropicalis* biofilms showed a decrease in terms of the number of culturable cells from 48 to 72 h, for strains

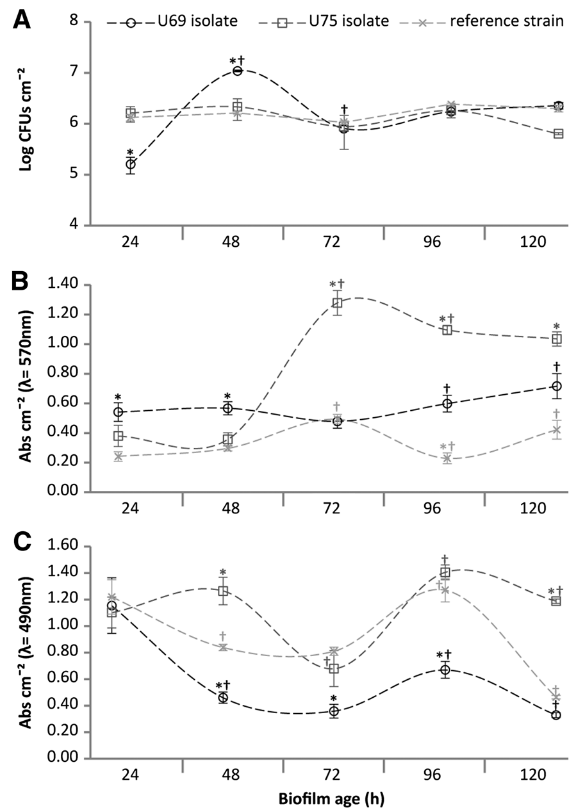

Fig. 1 *Candida tropicalis* biofilm characterization; **a** number of culturable yeasts determined by colony-forming units; **b** biofilm biomass quantification by crystal violet; **c** in situ mitochondrial biofilm metabolic activity by XTT. *Statistical difference between strains ($p < 0.05$); †Statistical differences between biofilms with different ages ($p < 0.05$)

U69 and U75, $p < 0.05$. Concerning *C. tropicalis* biofilm biomass, it is possible to observe (Fig. 1b) that there were some differences between the strains and between biofilms of different ages. The isolate U69 presented the highest biofilm biomass ($p = 0.01$) at 24 h (0.54 abs/cm^{-2}) and 48 h (0.57 abs/cm^{-2}) corresponding to a moderate biofilm, but for 72-h biofilms, the highest biomass ($p = 0.01$) was attained by strain U75 (1.28 abs/cm^{-2}; high biofilm biomass), highlighting the differences in the behaviour of the three strains. The analysis of in situ biofilm (Fig. 1c) indicated that all strains independently of time points showed high metabolic activity (above 0.2 abs/cm^{-2}). Although there were some differences in the first time points, there was a pattern of activity among the different strains after 72 h; namely, there was a significant increase ($p < 0.05$) from 72 to 96 h and a

decrease from 96 to 120 h. Until 72 h, the different strains presented distinct behaviours, while *C. tropicalis* reference strain and U69 presented a decrease in activity from 24 h (1.15 abs/cm^{-2}) until 72 h (0.36 abs/cm^{-2}) and strain U75 presented a slight increase from 24 h (1.10 abs/cm^{-2}) to 48 h (1.27 abs/cm^{-2}) and a decrease from 48 to 72 h (0.68 abs/cm^{-2}).

Candida tropicalis SAP Gene Expression

The primers *SAPT1-4* and *ACT1* demonstrated efficiency and similarity in titration experiments using cDNA of *C. tropicalis* ATCC 750 both planktonic and biofilm cells (23.6–88.3 ng) in serial log10 dilutions (data not shown). Analysing *C. tropicalis SAP* gene expression (Table 1), it can be noticed that *C. tropicalis* suspended cells grown in AU were not able to express *SAPT1* gene. However, cells of 48-h biofilms of strains U69 and ATCC 750, when grown in the sessile form, were able to express that gene with 0.02 and 0.01 of relative *SAPT1* gene expression, respectively. As regards *SAPT2* gene, it was expressed by *C. tropicalis* planktonic and biofilm cells, although at low levels. In opposition to the other *SAPT* genes, *SAPT3* was expressed in all conditions (planktonic and biofilm), except by ATCC 750 120-h biofilm cells (<0.01). Interestingly, the amount of this *SAP* gene expressed by planktonic cells is much higher than the amount expressed by biofilm cells. Interestingly, *SAPT4* was only expressed by the reference strain and in few situations (48-h biofilms with 0.02 relative *SAPT4* gene expression).

Discussion

Candida tropicalis is emerging as a cause of infection in hospitalized patients [3, 4, 8, 15, 18] being involved in nosocomial candidemia and candiduria [9, 18]. Urinary catheter use is the principal determining factor for the emergence of urinary yeast infections [28]. However, the information on the behaviour of *Candida* species under human body conditions such as urine is still limited [12, 21]. In the present study, *C. tropicalis* biofilm formation in AU was investigated. In accordance with other studies [7, 11, 14, 21], the strains of *C. tropicalis* assayed here were able to form biofilms in the presence of AU (Fig. 1), although in a strain- and time-dependent way. Similarly, a study by Jain et al. [21] with *C. albicans, C. glabrata* and *C. tropicalis,* using AU and RPMI 1640, showed that biofilm formation varied considerably among isolates under both growth conditions.

The most common methods for analysing *Candida* biofilm development include quantification of biomass production using crystal violet (CV) stain, quantification of the metabolic activity of viable embedded biofilm cells based on reduction in tetrazolium salt XTT to formazan dye and the counting of viable cells (in terms of CFUs). These methods (CV, XTT and CFUs) serve as complementary procedures for the study of *Candida* biofilm, showing different

Table 1 Detection by quantitative RT-PCR of secreted aspartyl proteinases (*SAPT1-4*) gene expression by planktonic and biofilm cells of *Candida tropicalis*

Strain (*n*)	Form	Range of relative expression of *SAPT* genes*			
		SAPT1	*SAPT2*	*SAPT3*	*SAPT4*
C. tropicalis (3)	Planktonic	<0.01	<0.01–0.01	9.74–25.31	<0.01
	Biofilm 24 h	<0.01	<0.01–0.03	0.06–2.35	<0.01
	Biofilm 48 h	<0.01–0.02	<0.01–0.07	0.30–20.95	<0.01–0.02
	Biofilm 72 h	<0.01	<0.01–0.01	0.01–1.09	<0.01
	Biofilm 96 h	<0.01	0.01–0.01	1.53–2.89	<0.01
	Biofilm 120 h	<0.01	<0.01	<0.01–1.15	<0.01

<0.01 indicates that gene expression was detected lower than 0.01

* Mean arbitrary messenger RNA transcript levels based upon triplicate measurements, presented as a percentage relative to the respective *ACT1* transcript level

information such as quantity of metabolic activity and total biomass compared to, for example, the counting of viable cells results [26, 29–32]. Observing the biofilm profile along time (Fig. 1), no consistent pattern can be noticed among the different strains. The only similarity among strains is an increase in the number of culturable cells and biofilm metabolic activity from 72- to 96-h biofilms. Variations among *C. tropicalis* strains concerning biofilm formation are expected due to the physiological differences between strains [24, 26]. Furthermore, as reported before, *C. tropicalis* species mature biofilms consist of a dense and heterogeneous network of yeast, pseudohyphae and hyphae and these forms are not always similar among *C. tropicalis* strains. Moreover, other studies reported that biofilm kinetics is strain dependent, mainly in *C. tropicalis* strains [11, 26, 28, 33].

For instance, in the present situation, U69 strain 24-h biofilm presented the lowest number of culturable yeasts (1.60×10^5 CFU cm^{-2}; $p = 0.01$), although showing the highest biofilm biomass (0.54 abs cm^{-2}; $p = 0.01$), considerate moderate biofilm forming [26]. However, in a previous work [12], using RPMI 1640 as growth medium, U69 biofilm biomass was similar to the other *C. tropicalis* strains, which highlights that biofilms are dependent on growth medium, carbohydrate supplementation and the nature of the colonized surface [11, 21, 24, 28].

It has been reported that culturable yeast cells and biofilm metabolic activity seem to be dependent on biofilm maturity with an increase in those parameters along biofilm development although the amount of retained product may vary between different cellular states, for example planktonic and biofilm [11, 26, 28, 34]. From the present results, it is not possible to establish a relation between those two parameters, which showed variations in biofilm metabolic activity along time; however, culturable yeast cells were constant (except to U69 isolate).

Furthermore, *C. tropicalis* biofilms show opposite kinetics (Fig. 1b, c) mainly at 24- and 72-h-old biofilms that at the moment of reaching the peaks of biomass production, the metabolic activity of the biofilms is lower and the inverse effect is also shown, corroborating with Marcos-Zambrano et al. [26]. *Candida tropicalis* biofilms have a thick extracellular matrix that can impair diffusion of nutrients and oxygen, leading to lower metabolic activity in the cells [18, 26, 28, 33, 34]. Thus, differences in biofilm matrix structure can explain the differences in metabolic activity between all time points assayed of biofilms and between *C. tropicalis* strains.

Concerning real-time PCR analysis (Table 1), the *SAPT* genes were expressed during *C. tropicalis* biofilm formation in the presence of AU. It has been widely reported that, during the adhesion and invasion processes of host tissues, *Candida* species are able to secrete hydrolytic enzymes that cause damage on host cells membrane integrity, leading to dysfunction or disruption of host structures [27, 35, 36]. Additionally, the expression of *SAPT* genes by planktonic *C. tropicalis* (*SAPT*1 to *SAPT*4) has also been demonstrated on the surface of fungal elements penetrating cells and tissues during disseminated infection and evading macrophages after yeast cells phagocytosis [27, 35, 37]. Although little is known about the contribution of *SAPT* genes to *Candida* biofilm formation, recent findings [7] showed the ability to express *SAPT* genes of *C. tropicalis* biofilms in AU, in the presence of human urinary bladder cells (TCC-SUP). Furthermore, research with *C. albicans* showed that this species adhered to abiotic surfaces or in biofilms producing more secreted *SAPs* than the planktonic cells [25, 38].

The *SAPT* gene expression by *C. tropicalis* grown in AU (in planktonic and biofilm forms) showed, in general, a higher level for *SAPT3* expression followed by lower levels of *SAPT2*, *SAPT1* and *SAPT4*. Only two strains (U69 and ATCC 750 strains) in 48-h biofilms were able to express *SAPT1,* but in planktonic form, this gene expression was not detected. These features were similar to those described by Silva et al. [27] when studying the expression profiles of *SAPT* genes by seven *C. tropicalis* strains in contact with reconstituted human oral epithelium.

Nailis et al. [39] found differences in *C. albicans* *SAP* gene expression between in vitro grown biofilms and in vivo model. In the present study, there is also a different gene expression among the different modes of growth. Curiously, *SAPT4* was only detected in ATCC 750 biofilms and at specific biofilm ages. Furthermore, the levels of *SAPT3* expression were more pronounced in planktonic cells than in biofilm cells. Other studies indicate that there is an optimum pH for *C. tropicalis*-secreted aspartic proteinases activity, thereby making *SAP* gene expression strain and substrate dependent [27, 35, 37]. Additionally, it is known that cells grown in biofilm form show

differences in metabolic activity when compared to those grown in planktonic mode. Maybe, this fact can be an explanation for the difference in *SAPT3* expression levels between planktonic and biofilm cells [7, 33]. This idea is enhanced when it was observed in previous research [7] with different ages (24 and 120 h) of *C. tropicalis* biofilms and their effect in human urinary bladder cells where there was a different gene expression among the different modes of growth. Also, there are only few studies reporting *C. tropicalis SAP* gene expression during the adhesion to human cells, and there is also limited knowledge about the role of these enzymes in *C. tropicalis* biofilms [7, 24, 33].

Conclusions

The present study showed that *C. tropicalis* strains assayed were able to form biofilms in silicone and in the presence of AU although in a strain- and time-dependent way, and *SAPT* genes are expressed during *C. tropicalis* biofilm formation. Nevertheless, *SAPT3* transcript presented the highest level of gene expression, regardless of biofilm age. However, more studies have to be performed to clarify whether these *SAP* genes are associated with biofilm development and *C. tropicalis* virulence potential.

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Author contributions MN conceived of the study, carried out the *C. tropicalis* biofilm studies, participated in the analysis of *SAP* gene expression and drafted the manuscript. SS carried out the analysis of *SAP* gene expression, performed the statistical analysis and helped to draft the manuscript. IRGC helped to draft the manuscript. JA participated in the design of the study and helped to draft the manuscript. MH participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

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