

Expression profile of genes associated with biofilm formation in *Candida* spp. isolates from patients with recurrent vulvovaginal candidiasis

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Abstract

The virulence of *Candida* spp. has been associated with the development of recurrent vulvovaginal candidiasis (RVVC). Among these virulence factors, biofilms represent a mechanism for fungal persistence in tissues, which could explain the recurrence of this condition. This study evaluated the expression profile of genes related to biofilm formation in *Candida* spp. isolates from patients with RVVC. These isolates were divided into two groups: 26 isolates from patients with $\leq$ seven episodes of VVC and 11 isolates from patients with $\geq$ eight episodes of VVC, each over a period of one year. The expression of the genes *ALS1*, *ALS3*, *BCR1*, *HWP1*, *EFG1*, *TEC1*, *ROB1*, *UME6*, *NRG1*, *ACE2*, *PGA10*, and *ACT1* was evaluated in both planktonic cells and their biofilm form using qPCR. A significant increase in the expression of genes associated with the adhesion phase (*ALS1* and *PGA10*) and the dispersion phase (*UME6*) was observed in planktonic cells. In biofilm cells, increased expression of the *ROB1* and *BCR1* genes and decreased expression of the *ACE2* gene were observed. Interestingly, the changes in expression were more significant in isolates from patients with more than eight episodes of VVC per year. These results suggest that the expression of genes related to biofilm formation is one of the most important virulence mechanisms of *Candida* spp. and appears to be associated with the number of VVC recurrences.

1. Introduction

Vulvovaginal candidiasis (VVC) is a yeast infection caused by *Candida* spp., affecting approximately 75% of the world's female population at least once in their lifetime [1]. There is also a phenomenon known as recurrent vulvovaginal candidiasis (RVVC), defined as three or more episodes of VVC in one year [2], and it is estimated that between 5% and 10% of women with VVC may develop the recurrent form [3]. Among the clinical manifestations of this disease, pruritus is reported as the most frequent symptom, generally accompanied by leukorrhea, pain, burning sensation, and inflammation [4]. RCVV is a multifactorial condition, where aspects such as changes in the composition of the vaginal microbiota (dysbiosis), hormonal changes, alterations in the immune response, underlying diseases such as diabetes, the use of broad-spectrum antibiotics or corticosteroids, and genetic predisposition are determinants that are associated with the development of this disease [5].

Candida spp. behaves as a commensal agent in the body's mucous membranes without causing damage; however, when the host-microorganism relationship is disrupted, this fungus activates its virulence factors to establish the pathological process [1]. Different *Candida* species have various virulence factors. *C. albicans* is the most studied species, and it has been found to have several virulence factors, including proteases, phospholipases, hemolysins, morphological changes (dimorphism), and biofilm development. This series of virulence mechanisms aims to overcome the body's defenses and establish an environment conducive to *C. albicans* proliferation and disease development [6]. Biofilms pose a constant challenge, as they represent a means by which *C. albicans* can persist on surfaces or tissues, evade the immune response, and resist the action of antifungals [7, 8].

The ability of *C. albicans* to form biofilms in the vaginal epithelium has been documented, as these structures have been observed in the vaginal mucosa of patients with RVVC, raising the possibility of disease persistence mediated by this virulence mechanism [9]. The expression of various genes associated with biofilm formation has been linked to the virulence of *C. albicans*; these include adhesion genes such as *ALS3*, which is also associated with resistance to drugs such as fluconazole [10]. Conversely, the absence of genes such as *RLM1* and *BCR1*, which mediate extracellular matrix synthesis and biofilm development, affects the virulence of this microorganism and its ability to infect tissues [11, 12].

Based on the above, this study evaluated the expression profile of various genes associated with the adhesion, maturation, and dispersion processes during biofilm development in *Candida* spp. isolates from patients with RVVC.

2. Materials and methods

2.1 Clinical Isolates of *Candida* spp.

Forty *Candida* spp. isolates were from patients with clinical and microbiological diagnoses of RVVC were included. The isolates comprised 37 *Candida albicans* and three *Clavispora lusitaniae* (formerly *Candida lusitaniae*). Additionally, the *C. albicans* isolates were divided into two groups according to the number of episodes reported by the patients: group I (26 isolates from women with $\leq$ seven episodes of VVC in one year) and group II (11 isolates from women with $\geq$ eight episodes of VVC). The isolates were identified using the Vitek®2 Compact system (BioMérieux, Inc., Durham, USA). The *C. albicans* strain SC5314 and the *C. parapsilosis* ATCC 22019 strain were also included as controls.

This study was approved by the Bioethics Committee for Human Research of the University Research Headquarters (SIU) of the Universidad de Antioquia, Medellín, Colombia (Act No. 21-28-935).

2.2 Planktonic Cell Culture

The 40 isolates, along with the control strains, were cultured on Sabouraud dextrose agar (SDA) supplemented with 1% penicillin/streptomycin and incubated for 24 hours at 37°C. After this incubation period, one colony was selected and subcultured in 5 mL of brain-heart Infusion (BHI) broth for 24 hours at 37°C with constant shaking. The cells were then used in their planktonic form.

2.3 Biofilm Formation Assay

The biofilm formation assay was conducted following the methodology described by Pierce *et al.* [13]. An inoculum of 1×10^6 cells/mL was prepared in RPMI 1640 medium supplemented with 4-morpholinopropanesulfonic acid (MOPS). Subsequently, 100 μ L of each standardized inoculum was plated into the wells of a 96-well flat-bottom culture plate and incubated for 48 hours at 37°C in a humidified environment. After this incubation period, each well was washed twice with 200 μ L of PBS to remove non-adherent cells that were not forming a biofilm.

2.4 RNA Extraction from *Candida* spp. Isolates in Planktonic Phase and Biofilms

RNA extraction from planktonic cells and biofilms was performed using the TRIzol-Chloroform method. Basically, 200 µL of TRIzol was added to an Eppendorf tube, followed by vortexing and the addition of 200 µL of chloroform. The mixture was centrifuged at 4500 rpm for 25 minutes at 4°C, and the supernatant was transferred to a new Eppendorf tube. Then, 200 µL of TRIzol and 200 µL of chloroform were added, followed by centrifugation at 4500 rpm for 25 minutes at 4°C. The resulting supernatant was transferred to an Eppendorf tube, and 400 µL of chloroform was added. After centrifugation at 4500 rpm for 25 minutes at 4°C, the supernatant was selected and transferred to another Eppendorf tube, and 600 µL of isopropanol was added. The mixture was incubated overnight at -80°C and then centrifuged again at 4500 rpm for 25 minutes at 4°C. The supernatant was then decanted, and 600 µL of 70% ethanol was added. The mixture was centrifuged once more at 4500 rpm for 20 minutes at 4°C. Finally, the supernatant was discarded, and the RNA was resuspended in 20 µL of nuclease-free water.

The extracted RNA was quantified using NanoDrop One (Thermo Fisher Scientific). Subsequently, it was treated with DNases, and complementary DNA was synthesized using the Applied Biosystems kit (4368814). Finally, the quantity and quality of the cDNA obtained were evaluated using NanoDrop One.

2.5 Gene Expression Assessment

The primer sequences are described in Table 1. Some sequences were designed de novo using the Primer Blast tool (NCBI). Real-time PCR reactions were performed using the ExcelTaq 2X Fast Q-PCR Master Mix kit, SYBR, without ROX (SMOBIO, TQ1200). The CFX96 Touch platform (Bio-Rad) was used. A final volume of 15 µL was used per reaction. The amplification protocol was as follows: an initial polymerase activation phase of 20 seconds at 95°C, followed by 40 cycles at 95°C for 15 seconds with a 30-second melting and extension phase. Melting curve analysis was also included. The relative expression of each gene was calculated using the $2^{-\Delta\Delta C_t}$ formula. The ACT1 gene was used as a normalizer.

Table 1
Primers used in real-time PCR.

Gene	Sequence 5'→ 3'	Reference
<i>ACE2</i>	F-TCAGCACTACCAGACACTGC R-GCAGTCGGAGATGGAGATGG	This study
<i>ALS1</i>	F-CAACAGGCACCTCAGCATCTAC R-CTCCACCAGTAACAGATCCACTAGTAA	Nailis <i>et al</i> [14].
<i>ALS3</i>	F-GTTGGGTTTGGCAGTGGAAC R-TACTACCGCTGTGACCACCT	This study
<i>BCR1</i>	F-AGGCATTCCCTGTTGTGGTT R-ACAAGCCCAAGTTCACATGC	This study
<i>EFG1</i>	F-CATCACAACCAGGTTCTACAACCAAT R-CTACTATTAGCAGCACCACCC	Nailis <i>et al</i> [14].
<i>HWP1</i>	F-GACCGTCTACCTGTGGGACAGT R-GCTCAACTTATTGCTATCGCTTATTACA	Nailis <i>et al</i> [14].
<i>NRG1</i>	F-ATCAGACCATCTCCAGGGCT R-TGGGTCTTTGCTTTGGGTGT	This study
<i>PGA10</i>	F-TCCCAGTATGGACATGGGGT R-GTGCCACTTCAGACGGTACA	This study
<i>ROB1</i>	F-CTGGATTCATCTCTTGTTTCACC R-TGTTGTTGGTTAAGGTGGACGTG	Nailis <i>et al</i> [14].
<i>TEC1</i>	F-ACTATCGGGGCTTGACTCGT R-CCAGAAAACGCATCACCCAC	This study
<i>UME6</i>	F-TCATTCAATCCTACTCGTCCACC R-CCAGATCCAGTAGCAGTGCTG	Nailis <i>et al</i> [14].
<i>ACT1</i>	F-TGGAAGCTGCTGGTATTGACC R-TGGATGGACCAGATTCGTCG	This study
F: Forward. R: Reverse		

2.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9.0 software (Dotmatics). Normality criteria were assessed, and based on the results obtained, parametric or non-parametric tests such as Student's *t*-test and the Mann-Whitney U test were applied, respectively. A *P*-value < 0.05 indicated statistically significant differences between the data.

3. Results

3.1 Expression of genes associated with the adhesion phase during *Candida* spp. biofilm formation

The genes *ALS1*, *ALS3*, *HWP1*, and *PGA10*, which are associated with the adhesion phase in *Candida* during biofilm formation, were evaluated. A significant increase in the expression of the *ALS1* and *PGA10* genes was observed in the planktonic phase compared to the biofilm phase of *C. albicans* isolates. Interestingly, expression levels were significantly higher in isolates from patients with $\geq$ eight episodes of VVC ($p < 0.01$) (Figs. 1a and 1d). In contrast, no differences were observed between planktonic and biofilm cells for the *ALS3* and *HWP1* genes (Figs. 1b and 1c).

3.2 Expression of genes associated with the maturation phase during *Candida* spp. biofilm formation

A significant increase in the expression of the *ROB1* and *BCR1* genes was observed during the maturation phase of biofilm formation in all *C. albicans* isolates from patients with VVC (Figs. 2a and 2b). Interestingly, *BCR1* gene expression was significantly higher in the biofilms of *C. albicans* isolates from patients with $\geq$ eight episodes of VVC compared to isolates with $\leq$ seven episodes of VVC ($p < 0.05$) (Fig. 2b). Regarding *EFG1* gene expression, a significant increase was observed only in the planktonic form of isolates from patients who experienced fewer than seven VVC episodes per year, similar to that observed with the *C. albicans* control strain (Fig. 2c). A significant increase in *ACE2* gene expression was also observed in all *C. albicans* isolates from patients with RVVC, with higher expression of this gene in isolates from patients who experienced more than eight VVC episodes per year (Fig. 2d). Finally, for the *TEC1* gene, no differences in expression were observed between the planktonic and biofilm forms in any of the *Candida* spp. isolates (Fig. 2e).

3.3 Expression of genes associated with the dispersion phase during *Candida* spp. biofilm formation

A significant increase in *UME6* gene expression was observed in all *Candida* spp. isolates during the planktonic phase, with no differences between groups according to the number of episodes (Fig. 3a). Finally, *NRG1* gene expression was significantly increased in both the planktonic and biofilm phases in *C. albicans* isolates from patients with eight or more episodes of VVC per year compared with *C. albicans* isolates from patients with fewer than seven episodes of VVC ($p < 0.05$) (Fig. 3b).

4. Discussion

The *Candida albicans* microorganism is part of the human microbiota, colonizing various parts of the body such as the skin and mucous membranes of the gastrointestinal, respiratory, and genitourinary tracts [15]. This fungal agent can produce various clinical presentations, ranging from localized superficial infections to complications such as invasive or disseminated infections. Infections caused by *Candida* spp. include mucocutaneous candidiasis, oropharyngeal candidiasis, candiduria, disseminated candidiasis, healthcare-associated infections due to colonization of medical devices, vulvovaginal candidiasis (VVC), and recurrent vulvovaginal candidiasis (RVVC) [1, 8, 15]. This fungus possesses various virulence factors that facilitate the processes of infection and invasion of tissues, including adhesion to surfaces, enzyme secretion (phospholipases, proteases, lipases, and hemolysins), morphological transition (dimorphism), and biofilm formation [6].

Biofilms represent one of the most important virulence factors in *Candida* spp., as this complex structure of yeast cells, hyphae, and extracellular matrix allows the fungus to resist the action of antifungal agents and evade the immune system. Consequently, biofilm-associated infections have a significant clinical impact, as biofilms enable fungal persistence on biotic or abiotic surfaces [16]. Biofilm formation involves a series of mechanisms mediated by several phases: i) adhesion, ii) proliferation and maturation, and iii) dispersion [17]. In this study, we evaluated the expression of various genes associated with each of these stages during biofilm formation in *Candida* spp. isolates from patients with recurrent vulvovaginal candidiasis (RVC).

During the adhesion phase, *Candida* spp. yeasts in their planktonic phase adhere to surfaces, forming a basal monolayer. Genes from the agglutinin-like sequence (ALS) family have been reported to encode glycoproteins expressed on the fungal cell surface that participate in adhesion processes and are also associated with pathogenicity mechanisms in *Candida* spp. [18]. In this study, the *ALS1* gene was found to have a higher expression during the planktonic phase, with a subsequent decrease in gene expression in biofilms formed after 48 hours. Previous studies have reported similar results, showing lower *ALS1* gene expression in biofilms formed after 48 hours [19]. Interestingly, in the present study, higher expression of the *ALS1* gene was observed in planktonic *C. albicans* isolates from patients with more than eight episodes of VVC in one year compared to *C. albicans* isolates from patients with fewer than seven episodes. Zhao *et al.* observed that deletion of the *ALS1* gene has effects including the generation of smaller yeast cells, a decrease in the ability to form germ tubes, and delays in disease progression in a mouse model [20]. La Bella *et al.* also reported that *ALS1* is a crucial gene for the colonization and pathogenesis processes of *C. albicans*; furthermore, the absence of this gene is associated with the formation of defective biofilms [21].

Regarding the *ALS3* gene, a higher expression was observed in the biofilms of *C. albicans* isolates from patients with more than eight episodes of VVC in this study. Consistent with our results, several studies have reported the importance of this gene; for example, in a study by Liu *et al.*, it was observed that the

glycoprotein encoded by this gene was necessary for biofilm formation and adhesion processes, where the *C. albicans* strain with a null genotype for *ALS3* showed variations in biofilm architecture and decreased biomass quantification [22]. In an additional study, Deng *et al.* investigated the relationship between *ALS3* expression, biofilm formation, and antifungal resistance, finding that *ALS3* expression was higher in *C. albicans* isolates that exhibited a greater capacity for biofilm formation [23].

The *HWP1* gene encodes an adherence protein expressed on the surface of *Candida* spp. hyphae, enhancing adherence and providing stability for biofilm formation [24]. Several studies have reported a variable *HWP1* expression in *C. albicans* isolates from women with vulvovaginal candidiasis, ranging from 25% to 75% [25, 26]. Interestingly, no differences in the expression of this gene were observed among the fungal clinical isolates evaluated in the present study.

The *PGA10* gene encodes a polypeptide structure belonging to a subset of proteins expressed on the fungal cell surface that plays a role in biofilm formation, development, and maintenance [27]. Similar to the expression of the *ALS1* gene, a significant increase in *PGA10* expression was observed in the planktonic phase of *C. albicans* isolates, with the highest expression detected in *Candida* isolates from patients with more than eight episodes of VVC. These results confirm that the *ALS1* and *PGA10* genes are primarily expressed during the adhesion process and subsequent biofilm development.

Additionally, the genes *ROB1*, *BCR1*, *TEC1*, *EFG1*, and *ACE2*, which are associated with the maturation phase, were evaluated. In the present study, an increased expression of the *ROB1* and *BCR1* genes was observed during the biofilm phase. The *ROB1* gene has been described as participating in the filamentation process, biofilm development, and commensalism [28]; furthermore, it has been described as a virulence factor of *C. albicans*, as confirmed in previous studies where deletion of this gene is associated with a decrease in fungal load and filamentation capacity [29, 30]. Conversely, the importance of the *BCR1* gene in biofilm formation processes has been described in both *in vitro* and *in vivo* models. Nobile *et al.* observed that *BCR1* is necessary for biofilm development and for the control of *ALS3*-mediated adherence [31]. At the same time, another study demonstrated that the absence of this gene is associated with the production of thinner biofilms, sparse growth patterns, and decreased extracellular matrix synthesis, thus reducing the virulence of *C. albicans* [32]. In the present study, higher *BCR1* expression was observed in the biofilms of *C. albicans* isolates from patients with more than eight episodes of VVC per year, compared with biofilms formed by *C. albicans* isolates from patients with fewer recurrences.

The *TEC1* and *EFG1* genes are associated with the synthesis and composition of the extracellular matrix and the morphological changes during biofilm development. Additionally, *TEC1* plays an important role in hyphal growth, architecture, and the arrangement of the extracellular matrix within the biofilm [33]. A previous study reported that the highest expression of the *TEC1* gene during *Candida* biofilm formation was observed at 12 hours, with expression decreasing significantly by 48 hours [19]. Panariello *et al.* observed that mutant strains defective in the *TEC1* and *EFG1* genes were less virulent and exhibited lower infectivity [34]. A relationship between the expression of the *EFG1* gene and the adhesion protein

Als1 has also been observed. Gregori *et al.* found a phenotype in *C. albicans* cells where *EFG1*-dependent *ALS1* expression was associated with a fungal protection mechanism that counteracted cell surface damage and participated in cell wall remodeling during antifungal stress [35]. Similarly, the contribution of *EFG1* to *C. albicans* virulence has been described, as this gene regulates the expression of several genes associated with hyphal formation. Korting *et al.* evaluated an *EFG1* mutant strain that lost the ability to form hyphae and showed a decreased capacity to damage epithelial cells and granulocytes [36]. It is important to note that, in this study, no changes in the expression of these two genes (*TEC1* and *EFG1*) were observed at 48 hours in either planktonic cells or biofilms; however, their expression could vary in the early stages of biofilm development.

ACE2 is a gene that plays an important role during *Candida* morphogenesis [37]. Mulhern *et al.* found that *ACE2* expression regulated filamentation and metabolic processes in *C. albicans*, and changes in *ACE2* expression can occur in both yeast and hyphal cells [38]. In our study, *ACE2* was significantly expressed during the planktonic phase in the evaluated clinical isolates.

Finally, regarding the dispersion phase, some cells detach from the biofilm and colonize other surfaces [17]. *UME6* and *NRG1* were the genes evaluated in this study, which are associated with this phase of biofilm development. *UME6* is a gene that is expressed in response to environmental stimuli, promoting hyphal growth [39]. This transcriptional regulator reduces dispersion, promoting biofilm formation and favoring the processes of adhesion and biogenesis of the biofilm's extracellular matrix [40]. In this study, higher *UME6* expression was observed in the planktonic stage, which is consistent with the findings reported by Do *et al.*, who observed higher expression of the *UME6-EFG1* complex under planktonic conditions [40]. The effects of *UME6* deletion, including loss of immunogenicity during colonization pr, have also been reported. Consequently, changes in *UME6* gene expression in *C. albicans* could make the difference between colonization and disease [41].

Dispersion influenced by *NRG1* expression allows the release of new daughter cells with greater virulence and environmental resistance compared with planktonic cells, enabling them to initiate new foci of colonization and infection [42]. Uppuluri *et al.* observed higher *NRG1* expression in biofilms formed after 24 hours, suggesting an important role in biofilm dispersion and contributing to the virulence of *C. albicans* [43]. In the present study, no differences in *NRG1* expression were observed during the planktonic or biofilm phases; however, a significant difference was observed in the biofilm phase of *C. albicans* clinical isolates from patients with more than eight episodes of VVC compared with isolates from patients with fewer recurrences.

All the findings described above could be correlated with a greater virulence of *C. albicans* isolates associated with a higher number of recurrences in patients.

It is important to note that further studies are needed to determine the expression of these genes at earlier stages of *Candida* spp. biofilm formation, in order to obtain a more accurate picture of the expression of these genes.

5. Conclusions

Biofilms present a complex challenge in the management of fungal infections. The expression of genes related to the formation of this virulence factor in *C. albicans* isolates from patients with RVVC provides new insights into biofilm virulence in relation to the number of recurrences. It was observed that some genes exhibited greater expression during the biofilm phase in isolates from patients with more than eight VVC episodes per year. Therefore, these isolates may exhibit greater adherence, more complex maturation, and a greater capacity for dispersion. These observations support the development of strategies for managing this pathology and the identification of targets of interest for the development of new agents or therapeutic strategies.

Declarations

Conflict of interest

The authors declare that they received no funding or grants during the preparation of this manuscript. The authors have no relevant financial or non-financial interests to disclose.

Ethical approval

This study was approved by the Bioethics Committee for Human Research of the University Research Headquarters (SIU) of the University of Antioquia, Medellín, Colombia (Act No. 21-28-935).

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Declaration

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Author Contribution

JFTG conceptualized the project, analyzed and interpreted the data, and wrote the first draft of the manuscript. JMCA analyzed the data and reviewed the manuscript. BT and CRE contributed to the project conception and reviewed the manuscript. AG analyzed and interpreted the data, supervised the project conceptualization, data analysis and interpretation, and reviewed the manuscript.

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Figures

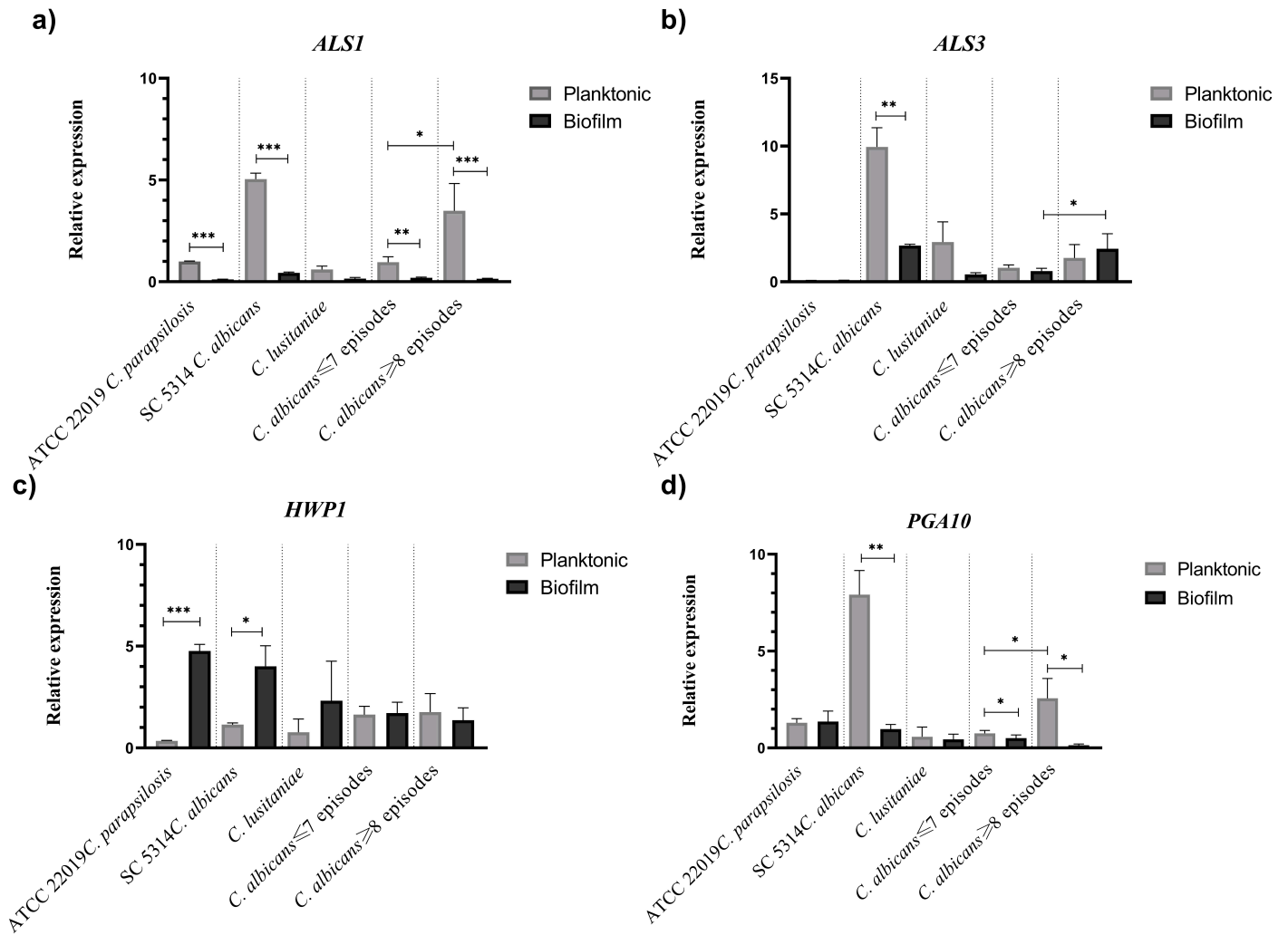


Figure 1

Expression of genes associated with the adhesion phase of biofilm formation. Relative expression of genes associated with the adhesion phase during biofilm formation in *Candida* spp. isolates from patients with RVVC. Results are expressed as mean $\pm$ standard error of the mean (SEM). a) *ALS1* gene; b) *ALS3* gene; c) *HWP1* gene; d) *PGA10* gene. A total of 40 *Candida* spp. isolates (37 *C. albicans* and 3 *C. lusitanae*) were evaluated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

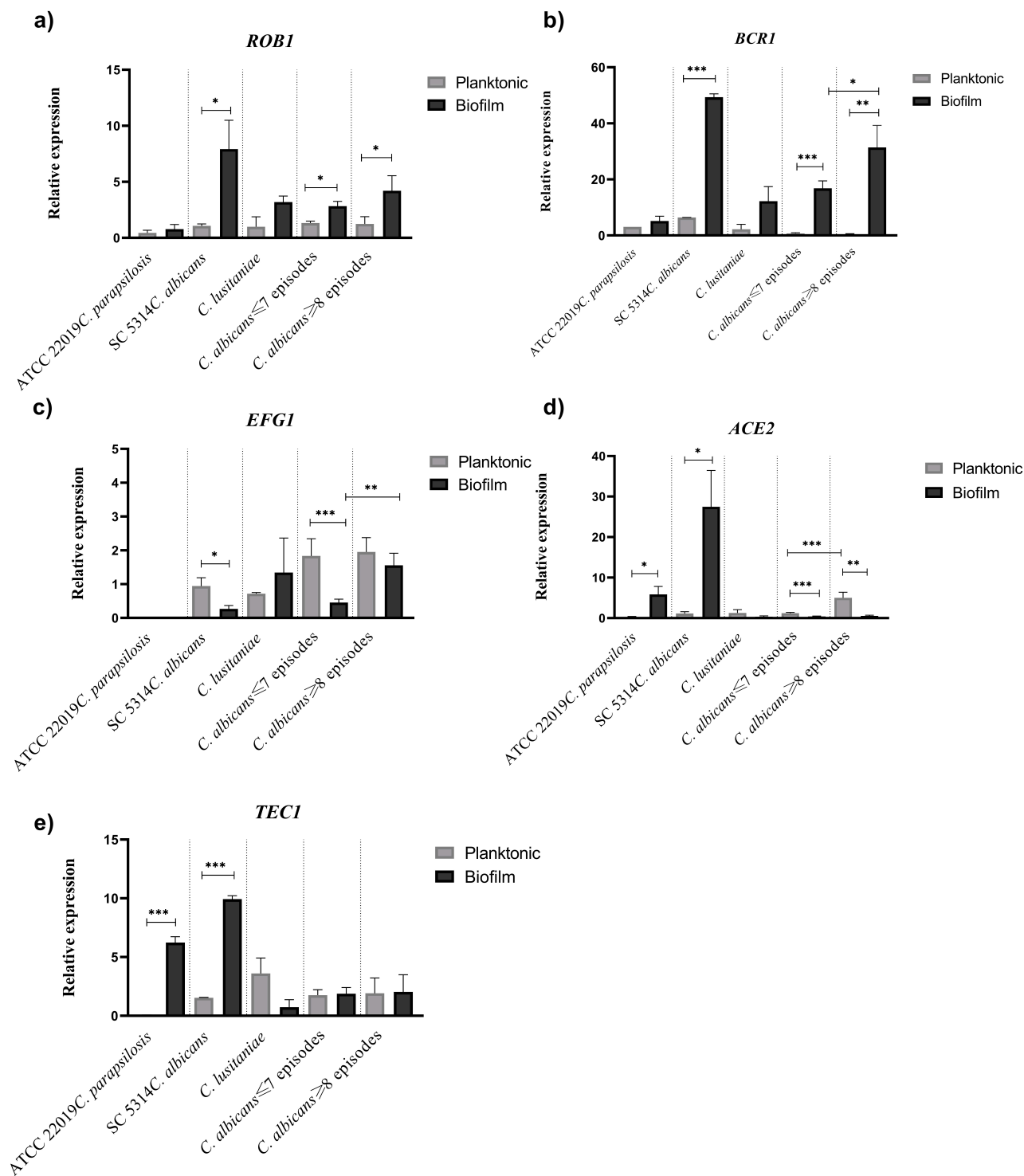


Figure 2

Expression of genes associated with the maturation phase of biofilm formation. Relative expression of genes associated with the maturation phase during biofilm formation in *Candida* spp. isolates from patients with RVVC. Results are expressed as mean $\pm$ standard error of the mean (SEM). a) *ROB1* gene; b) *BCR1* gene; c) *EFG1* gene; d) *ACE2* gene; e) *TEC1* gene. A total of 40 *Candida* spp. isolates (37 *C. albicans* and 3 *C. lusitanae*) were evaluated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

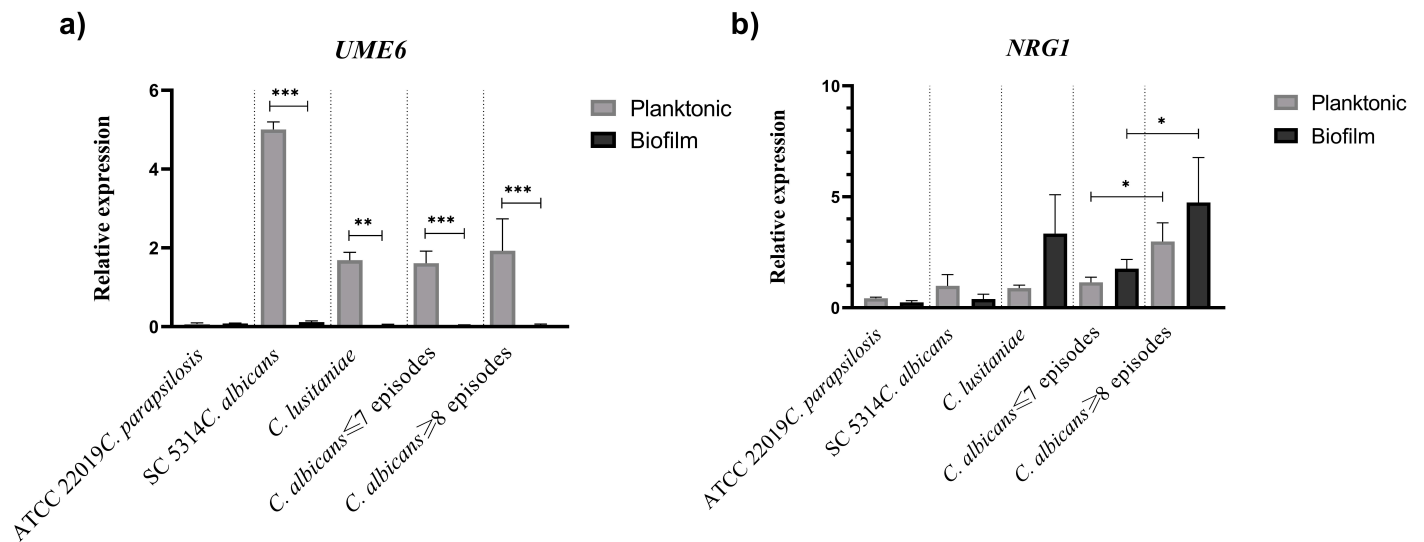


Figure 3

Expression of genes associated with the dispersion phase of biofilm formation. Relative expression of genes associated with the dispersion phase during biofilm formation in *Candida* spp. isolates from patients with RVVC. Results are expressed as mean $\pm$ standard error of the mean (SEM). a) *UME6* gene; b) *NRG1* gene. A total of 40 *Candida* spp. isolates (37 *C. albicans* and 3 *C. lusitanae*) were evaluated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.