Infection and Drug Resistance

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ORIGINAL RESEARCH

Sub-Therapeutic Concentrations of Hexetidine Induce Virulence Gene Expression in *Candida albicans*

Banu Uygun-Can, Tanju Kadir

Department of Microbiology, Dental Faculty, Marmara University, Istanbul, Turkey

Correspondence: Banu Uygun-Can, Email drbanucan@gmail.com

Purpose: Intraoral concentrations of antimicrobial agents such as hexetidine (HEX) using alternative antimycotics fluctuate significantly due to the dynamics in the oral cavity, which can affect both the virulence capacity and the outcome of antimycotic therapy in the management of denture stomatitis-associated oral candidiasis.

Methods: This study was carried out to evaluate the impact of different sub-therapeutic HEX concentrations (1/100, 1/200) for different times (0.5, 2, 24, 48 h) on the expression levels of the virulence (*hwp1, plb1, plb2, sap4, sap5, sap6*) and drug-resistance (*cdr1, cdr2, mdr1, erg11*) genes of *Candida albicans* SC5314. Total RNA was extracted immediately after antimicrobial exposure. The qRT-PCR data were performed using the comparative $2^{-\Delta\Delta CT}$ method to calculate the relative expression of the target genes after treatment to HEX concentrations, standardized to the actin housekeeping gene.

Results: Expression levels of the *hwp1* and *plb1* genes decrease after exposure to HEX to higher concentrations at 0.5 h, while the expression level of the *plb2* gene increases. Only the expression of the *erg11* gene showed a significant decrease within the resistance genes at 0.5 h. *Sap5, plb2*, and *mdr1* are overexpressed at 48 h. Sub-therapeutic levels of HEX affect the virulence and drug-resistance genes of *C. albicans*.

Conclusion: These results give an idea of future strategies for developing and using HEX as an adjunctive therapy for oral candidiasis. The innovation and clinical relevance of our research lie in its exploration of how low doses of HEX influence gene expression over varying time periods, potentially paving the way for alternative therapeutic strategies.

Keywords: antimicrobial agents, hexetidine, virulence genes, drug-resistance genes, oral candidiasis, *Candida albicans*

Introduction

Candida albicans and Candida spp. species are recognized as key components of the normal microbial flora present in the oral cavity, digestive system, and vagina of a wide range of healthy individuals. Immune deficiencies can disrupt the balance between *C. albicans* and other components of the normal biota. In such cases, the commensal *Candida* yeasts can turn into opportunistic pathogens within the host. The ability to switch between yeast and filamentous forms, known for its shape flexibility, is one of the most well-known pathogenic traits of the dimorphic fungus *C. albicans*. Additionally, several features of *C. albicans* are considered essential pathogenic mechanisms, including adhesion, invasion, secretion of hydrolytic enzymes, and biofilm formation.[1–3]

Candida albicans is an opportunistic pathogen frequently isolated from the oral cavity, responsible for causing various types of oral candidiasis, especially in individuals with compromised or weakened immune systems. Additionally, the rising use of antibiotics, hormones, antitumor drugs, and oral biomaterials like dentures has contributed to a yearly increase in oral candidiasis cases. In elderly individuals who wear dentures, the most common form of oral candidiasis is *Candida*-associated denture stomatitis.[4,5]

Polyene drugs can act as fungicidal agents, but they are often toxic to the host, while azoles are fungistatic, making fungi more likely to develop resistance.[6] Antifungal azoles like fluconazole are commonly used for treatment or prevention, and

Received: 31 October 2024
Accepted: 30 January 2025
Published: 14 February 2025

mouthrinses containing hexetidine (HEX) have been favored as adjunctive therapies or alternatives to traditional antifungal treatments due to the increasing resistance observed.[7] Several notable studies have reported that due to the diluting effect of saliva and the cleansing action of oral muscles, intraoral concentrations of antifungal agents decrease significantly, potentially resulting in sub-therapeutic drug levels and diminishing their therapeutic effectiveness.[8–10]

Previous reports have indicated that many non-antibiotic antimicrobial agents exhibit antiadhesive properties even at concentrations below their therapeutic levels, and also at concentrations lower than those required for complete substrate coverage. Since the adhesion of microorganisms to epithelial cells is a surface-level process and there is rapid clearance by saliva, it is plausible that the prolonged antiadhesive effects may be partially attributed to the retention of low concentrations of HEX on the surface of epithelial cells. Ongoing studies are focused on determining how HEX interacts with epithelial cell membranes.[11]

Numerous studies have reported that under such conditions, *Candida* behavior and its virulence factors are also impacted.[8] For instance, in vitrostudies conducted by several researchers[8–10,12,13] demonstrated that brief exposure of *C. albicans* to sub-minimal inhibitory concentrations of certain oral antiseptics significantly reduced germ tube formation, cell surface hydrophobicity, and enzymatic activities such as phospholipase and proteinase. All of these studies utilized phenotypic methods, but none examined the expression of genes responsible for virulence. Pathogenic *C. albicans* possesses 10 secreted aspartic protease (*sap*) isoenzymes, with *saps 4–6* playing a crucial role in mediating fungal hyphae invasion by penetrating tissues. According to the data, *saps 4–6* facilitate *C. albicans* hyphal penetration by hydrolyzing surface proteins, creating openings in the mucosal cells of the host. The main targets of *saps 4–6* are integrins located on the outer layer of host epithelial cells. These proteases bind to integrins, creating spaces for the fungal penetration process. This mechanism, referred to as the Trojan horse mechanisms, can induce apoptosis in host epithelial cells through the intralysosomal proteolytic activity of *saps 4–6*.[1] Consequently, the primary aim of this study was to analyze the genes that encode hyphal wall protein (*hwp1*), proteinase (*sap4, sap5, sap6*), and phospholipase (*plb1 and plb2*).

Due to inadequate treatment and recurrent infections, resistance to antifungal azoles in *C. albicans* has been observed to increase.[14–16] The most crucial genes involved in *C. albicans* drug resistance are (i) *cdr1* and *cdr2*, which encode multidrug efflux transporters from the ATP-binding cassette (ABC) transporter family, (ii) *erg11*, a gene encoding sterol 14 α-demethylase, the drug target enzyme, and (iii) *mdr1*, a major facilitator transporter gene.[17,18] Resistance can arise from changes in drug accumulation within the cell, alterations in membrane sterol composition, mutations in *erg11*, or changes in its function.[19] Therefore, the secondary objective of this study was to analyze the drug resistance genes (*cdr1, cdr2, mdr1*, and *erg11*) in *C. albicans* following brief exposure to sub-therapeutic concentrations of HEX.

The hypothesis of this study is that the expression of virulence and resistance genes in *C. albicans* will be progressively influenced by long-term fluctuations in the oral environment. The novelty and clinical importance of our research lies in its potential to open avenues for alternative treatment methods by examining the effects of low doses of HEX on gene expression over varying durations.

Materials and Methods

Strain

Candida albicans SC5314 (from our laboratory stock collection) stored in YEPD that contains 10% glycerol at −80°C. Fresh culture was used.

Antiseptic Agent

The preparation of hexetidine (HEX, 0.1%) was performed by adding 0.01 mL of 1% HEX (Sigma) to 0.09 mL of PBS, as previously described.[13] Sub-therapeutic concentrations (STC) (1/100, 1/200) of antiseptic agent were prepared by adding a therapeutic concentration of 10 µL from antiseptic agent to tubes containing 0.99 and 1.99 mL of PBS, 20 ppm and 10 ppm, respectively. In this study, the therapeutic dose for HEX (0.1%) was not used, because this dose was going to kill the microorganism[13] (Figure 1).

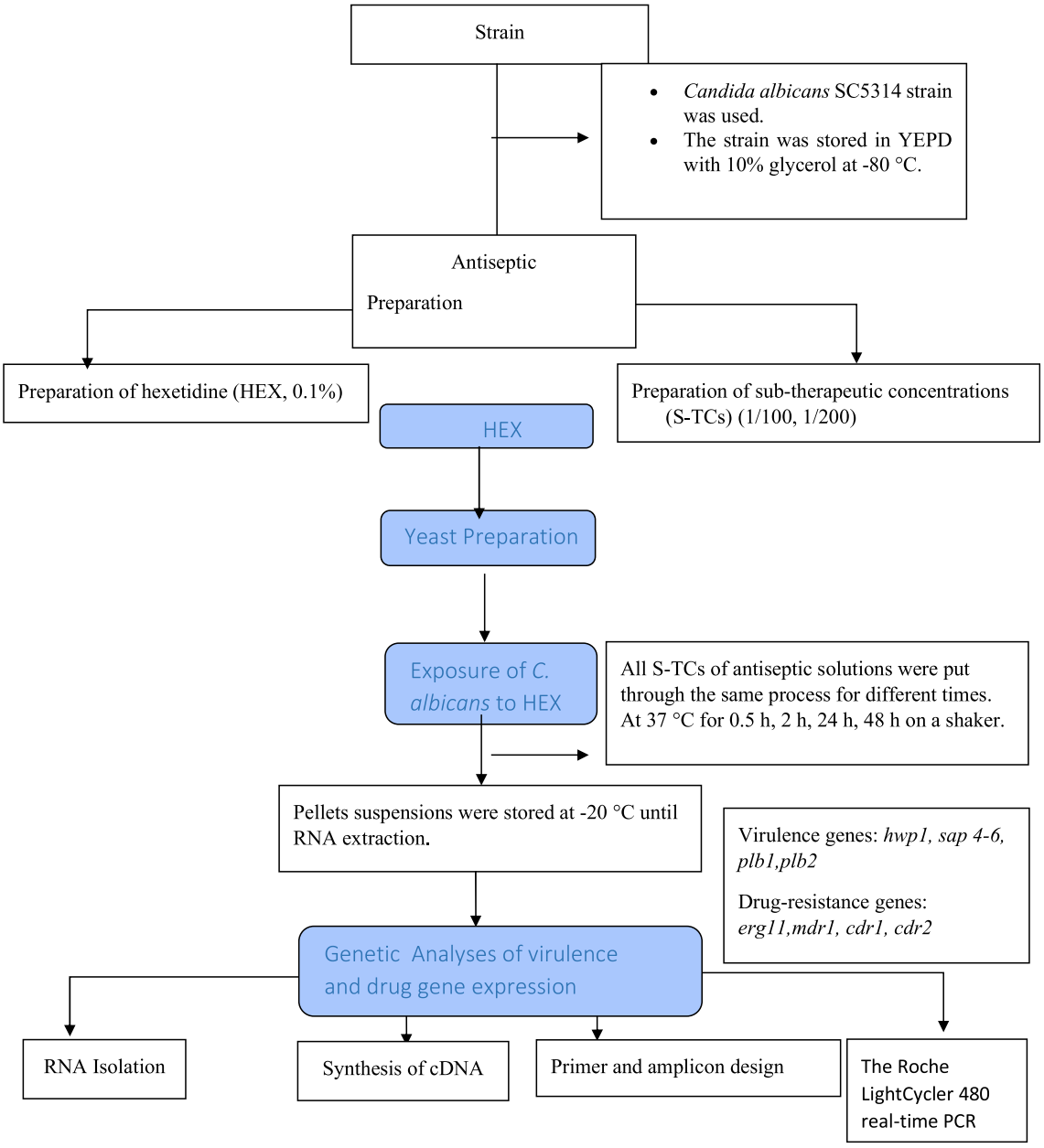


Figure 1 Overall schematic diagram representing the workflow.

Preparation of the Yeast Suspension

C. albicans SC5314 was cultured in YEPD broth (yeast extract at 10 g, peptone at 20 g, and dextrose at 20 g/liter) at 30° C for 24 hours. After culture for 24 hours in YEPD (at 30°C), cells were inoculated in YEPD, 200 mL and brought to the starting concentration of 2×104 cells per mL. Agitation was used to grow cultures overnight at 30°C, reaching 600 nm (OD600) of 0.1 optical density and growing in logarithmic-phase OD600=0.8–1 (approximately 1×107 cells mL−1).[19]

Exposure of C. albicans SC5314 to an Antiseptic Agent

Three tubes of cell suspension (10^7 yeast/mL) of the *C. albicans* SC5314 isolate were prepared in 1 mL of buffered phosphate saline (PBS). The tubes were centrifuged at 3000×g for 10 minutes. After the supernatant was completely decanted, all yeast pellets were resuspended in 1 mL of sterile PBS (control) and 1 mL of PBS/HEX. Each antiseptic solution underwent the same procedure at different time intervals. The tubes were incubated at 37°C for 0.5, 2, 24, and 48

https://doi.org/10.2147/IDR.S498943

hours on a shaker. Subsequently, two suspension and centrifugation cycles with sterile PBS were performed for 10 minutes at 4°C at 3000×g to thoroughly remove the supernatant containing antiseptic. After removing the supernatant, two additional washes were conducted, and the pellets were resuspended in 1 mL of sterile PBS to prevent any potential carry-over effects of the antiseptic.[20] These pellet suspensions were then stored at −20°C until RNA extraction.

Analysis of Gene Expression

Reverse transcription combined with polymerase chain reaction (RT-PCR) has been shown to be a highly effective technique for quantifying gene expression. This method reduces potential errors associated with post-PCR processing of PCR products, thereby increasing accuracy and minimizing the risk of carryover contamination.[21] In our study, we designed an assay that measures the expression of resistance genes at the mRNA level using RT-PCR.

The comparative ΔCt method was employed, enabling a relatively quantitative calculation of the sample template. This method enhances the output by eliminating the need for standard curves to determine expression levels, using an active reference control for comparison instead.[21,22]

RNA Isolation, cDNA Synthesis, and Quantification

The general extraction of RNA from *C. albicans* SC5314 strain was performed with a commercially available Bio-Speedy® RNA-TRiRegular (Bioeksen, Turkey), consisting of a solution of phenol and guanidine isothiocyanate solution, according to the manufacturer's instructions.

First-strand cDNA synthesis was carried out using the Biospeedy cDNA synthesis kit according to the manufacturer's instructions (Bioeksen, Turkey). Briefly, the first step of the reaction was performed with an incubation of 300 ng of total RNA in 6 µL RNase-free water and 2 µL oligo dT at 70°C for 10 min. The reaction mix was prepared with 4 µL 5X speedy reaction mix, 1 µL dNTP mix, 1 µL reverse transcriptase, 14 µL RNAse water, and 8 µL of the RNA mix. The final conditions of cDNA synthesis were 37°C for 60 min.

For the specific and efficient amplification of PCR products, it is very crucial to determine the target sequence and design the primers specific to these sequences. The sequences of the target genes were obtained from the NCBI DNA database (https://www.ncbi.nlm.nih.gov) for primer design, Primer-Blast was used to pick specific primers for our target genes listed in Table 1. In silico analysis of primers was performed using the Primer3 software (v.0.4.0), and those containing internal structures (ie, hairpins and formation of primer dimers, according to the software) in minimal amounts were chosen for qPCR analysis.

For successful and efficient amplification of PCR products, it is essential to accurately identify the target sequences and design primers specific to those sequences. The target gene sequences were retrieved from the NCBI DNA database (https://www.ncbi.nlm.nih.gov), and Primer-Blast was utilized to select primers specific to the target genes, as listed in Table 1. An in silico analysis of the primers was conducted using Primer3 software (v.0.4.0). Primers with minimal internal structures, such as hairpins and primer dimers, as predicted by the software, were selected for use in qPCR analysis.

Table 1 Primer Sets and Their Targets

Name	Primer	Oligonucleotide Sequence (5′ to 3′)	Position	Product (bp)	Tm (°C)
ACT1	Forward	TGCTGAACGTATGCAAAAGG	882–901	186	51
ACT1	Reverse	TGAACAATGGATGGACCAGA	1048–1067		
cdr1	Forward	CATGGTCAAGCCATTTTGTG	3148–3167	200	51
cdr1	Reverse	ATCCATTCTGCTGGATTTGC	3328–3347		
cdr2	Forward	GAGAAAGTTAGCTGATCAT	3142–3161	199	51
cdr2	Reverse	TGCTTCCTTAGGACATGGA	3322–3341		
erg11	Forward	TACTGCTGCTGCCAAAGCTA	1284–1303	278	53
erg11	Reverse	CCCAAATGATTTCTGCTGGT	1542–1561		

(*Continued*)

Table 1 (Continued).

Name	Primer	Oligonucleotide Sequence (5′ to 3′)	Position	Product (bp)	Tm (°C)
hwp1	Forward	TCTACTGCTCCAGCCACTGA	1066–1085	248	51
hwp1	Reverse	CCAGCAGGAATTGTTTCCAT	1294–1313		
mdr1	Forward	CAAATTCCCACTGCTTTGGT	592–611	148	51
mdr1	Reverse	CGGCTAACCCAACTGGTAAA	720–739		
plb1	Forward	ACTTCCAAAGCCACCAAATG	704–723	276	51
plb1	Reverse	TGATAGACAACACCGCTTCG	960–979		
plb2	Forward	AAGGGAACCAAACACTGTGC	747–766	290	53
plb2	Reverse	GGTCGAGGGTGAATTTGCTA	1017–1036		
sap4	Forward	AATGATGTGGGCAAAAGAGG	208–227	155	51
sap4	Reverse	ACGGCATTTGAATCTGGAAC	343–362		
sap5	Forward	ATTTCCCGTCGATGAGACTG	183–202	248	53
sap5	Reverse	CTGGGGAATAAGAACCAGCA	411–430		
sap6	Forward	GTCAACGCTGGTGTCCTCTT	859–878	197	55
sap6	Reverse	GCAGGAACGGAGATCTTGAG	1036–1055		

The Roche LightCycler 480 real-time PCR detection system (Roche, Germany) was utilized to assess resistance gene expression profiles at the mRNA level. Each 20-µL reaction mixture contained 4 µL of reverse-transcribed cDNA, 10 µL of EvaGreen qPCR pre-Mix (Bioeksen, Turkey), 500 nM of each primer, and nuclease-free water. The Bio-Speedy® EvaGreen qPCR preMix included Eva Green Dye® as the fluorescent dye, a one-fusion DNA polymerase, a dNTP blend with dUTP to prevent false positives, and optimized buffer components. The presence of EvaGreen dye in the reaction mix was expected to result in less non-target amplification compared to the more commonly used SYBR Green dye. Primer efficiency and amplicon specificity were verified through serial dilution of cDNA and melting curve analysis, respectively. Detection and amplification were performed using the Roche LightCycler 480 system. The protocol began with an initial denaturation step at 95°C for 10 minutes, followed by 40 amplification cycles: 15 seconds at 95°C, 1 minute at 49–50°C (depending on the annealing temperature), and 30 seconds at 72°C.

In the melting curve analysis, the products were heated to 95°C for 1 minute, then cooled to 65°C before being gradually reheated to 95°C, while fluorescence was monitored throughout the process. To ensure the reliability of the experiments, negative controls such as no-template control (NTC) and no-reverse transcriptase control (NRTC) were routinely included to detect any potential sources of contamination and to prevent the amplification of genomic DNA contaminants in each assay. None of the negative controls produced consistent Ct values after 40 qPCR cycles (data not shown). Changes in the relative mRNA levels were analyzed using the comparative critical threshold (2−ΔΔCT relative expression) qPCR method as described by Livak.[22] Amplifications of treated and untreated samples (positive controls) were conducted in separate wells. Relative quantification was carried out by comparing the expression of the target genes to that of the housekeeping gene (*ACT1*), which served as a reference for normalizing RNA input and the levels of all stress and virulence genes. The experiments were repeated three times for consistency.

Statistical Analysis

Bivariate correlation analyses were conducted using MINITAB (Minitab Ltd., England), with correlations assessed through the Pearson method. Statistical significance was set at $p < 0.05$. Expression levels of various virulence genes (*hwp1, sap4, sap5, sap6, plb1*, and *plb2*) and resistance genes (*erg11, cdr1, cdr2* and *mdr1*) in *C. albicans* were measured at 0.5, 2, 24, and 48 hours at different antiseptic concentrations – 10 ppm (1/200) and 20 ppm (1/100) – using qRT-PCR.

Relative quantification of target gene expression was calculated after normalization to *ACT1* under different experimental conditions. The amplification efficiency of each target gene was 1.01 ± 0.05. Results were expressed as nFold (2 −ΔΔCT), with values >1 indicating overexpression and values <1 indicating underexpression of target genes in response to antiseptic exposure at different incubation times.

Results

C. albicans Gene Expressions

The amount of change can be seen from Figure 2.

Expression levels of the virulence gene *hwp1* are significantly reduced compared to control (0.002) after exposure to HEX exposure at 0.5 h (Figure 2), *hwp1* is down-regulated at 24 h and 48 h (Figure 2) ($r>0.8$, $p<0.01$). After 2 hours, the upregulated for all concentrations (0.36–0.18) but was more upregulated at 10 ppm, compared to the control (0.038) (Figure 2).

Sap4 is up-regulated at 24 h, regularly increased by concentraions ($r>0.6$, $p<0.05$) (Figure 2). After 30 minutes, there was a slight decrease while this expression increased for 2 and 48 hours.

At 48 h, *sap5* showed an increase in expression (Figure 2). After 30 minutes, for 10 ppm, there is a little increase first, but then for 20 ppm, it significantly decreased ($r>0.8$, $p<0.05$). For 2 h, at all concentrations, it significantly increased ($r>0.6$, $p<0.05$) (Figure 2).

Sap6 expression levels are down-regulated after 30 minutes, while they are significantly up-regulated for the other times, at all concentrations of HEX (Figure 2).

Plb1 expression levels decrease after exposure to HEX at 0.5 h (Figure 2) and also at 10 ppm at 48 h. *plb1* is up-regulated for 2, 24 h (Figure 2) and at 48 h in all concentrations of HEX. For only 0.5 h and at 20 ppm concentration, it decreased significantly decreased ($r>0.6$, $p<0.05$) (Figure 2).

Plb2 gene expression levels are significant after HEX exposure at 0.5 (Figure 2) and 48 h regularly by concentrations ($r>0.6$, $p<0.05$). After 2 h, the first increased and then decreased, while controversial at 24 h (Figure 2).

At 0.5 h, only the expression of the *erg11* gene showed a significant decrease within the resistance genes ($r>0.6$, $p<0.05$) (Figure 2). At 24 and 48 h, it decreased at all concentrations, as well as (Figure 2).

Cdr1 is upregulated for 30 minutes and 2 h. For 24 and 48 h, it is significantly down-regulated at all concentrations of HEX. ($r>0.6$, $p<0.05$) (Figure 2).

Cdr2 is upregulated at 30 minutes. For 2 and 24 h, it is upregulated, while after 48 h it is down-regulated about 10-fold by first concentration, controversial by 20 ppm again up-regulated a little more (Figure 2).

Mdr1 showed a significant increase in expression at 2, 24, and 48 h (Figure 2). For only a 20-ppm concentration at 30 minutes, it was down-regulated approximately five-fold ($r>0.8$, $p<0.05$) (Figure 2).

Discussion

The rising morbidity and mortality rates associated with *Candida* infections in recent years have led researchers to focus on *Candida* virulence factors, prompting the development of various in vitro tests to investigate the role these factors play in fungal pathogenesis. Animal models of *Candida* infection have demonstrated the significance of *Candida* virulence factors and host defenses while also helping to explore new treatment alternatives.[23–25]

The initial stage of *Candida* infection is known to involve the adhesion of *Candida* to host cell surfaces. *Hwp1* is one of the key genes that mediates adhesion to mucosal surfaces[26] and is highly expressed in germ tubes during the early phase of biofilm formation.[27,28] Additionally, *C. albicans* possesses gene families that encode hydrolytic enzymes involved in infections, such as secreted aspartyl proteinases (*Saps*) and phospholipase (*Pl*) gene families.[29]

Although numerous antifungal agents, both topical and systemic, are available for the treatment of oral candidiasis, antiseptic mouthwashes can also serve as alternatives or adjuncts to traditional antifungal treatments.[7,30] Prolonged antifungal use, however, has led to a significant increase in the prevalence of antifungal resistance, complicating infection management.[31]

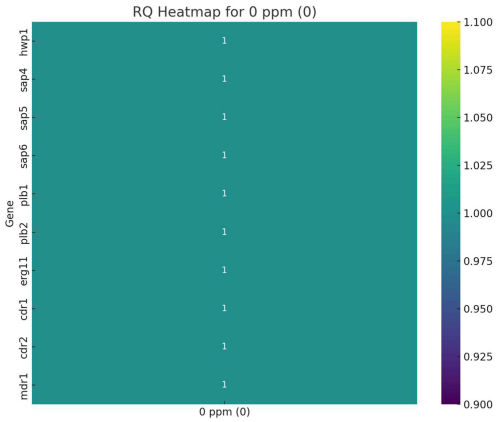
In recent years, many studies have uncovered the molecular mechanisms underlying drug resistance in yeast.[32,33] In this regard, the ATP-binding cassette transporters, specifically the *cdr*,[34] *mdr1*,[35] and *erg11*[36] genes, have been shown to play crucial roles in mediating *C. albicans* resistance to azole antifungal agents. Therefore, understanding these mechanisms is essential for developing more effective therapeutic strategies to combat antifungal resistance.

Most antiseptic agents are eliminated from the oral cavity within the first hour due to the diluting action of saliva and the cleansing effect of oral musculature, which diminishes their therapeutic efficacy.[9] This raises the question of whether

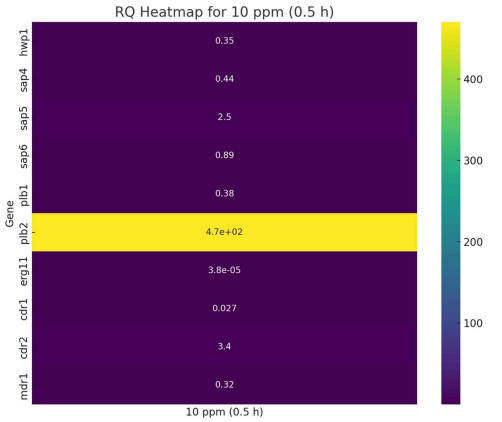
RQ Heatmaps for Gene Expression

Heatmap of Gene Expression Changes Across Time and Concentrations

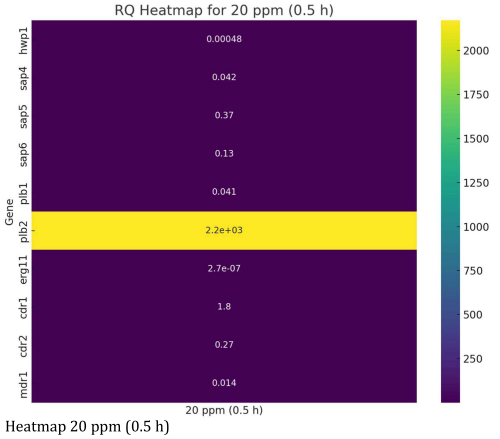
The heatmap below visualizes the changes in gene expression across different times and concentrations of HEX treatment. The data has been normalized to better highlight the relative differences.



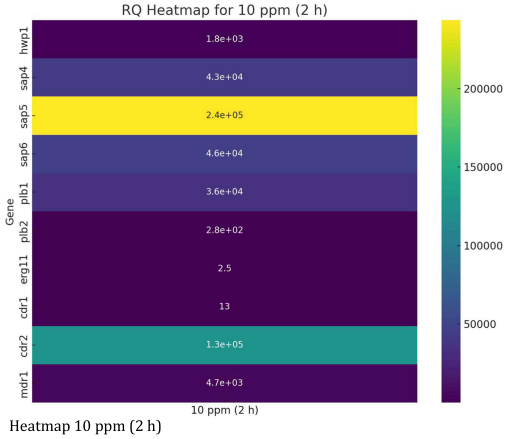
Heatmap 0 ppm (0)



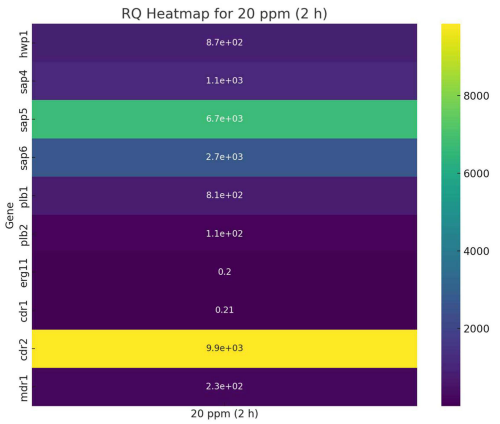
Heatmap 10 ppm (0.5 h)



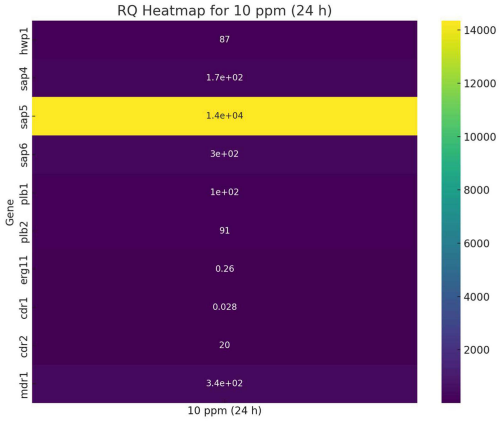
Heatmap 20 ppm (0.5 h)



Heatmap 10 ppm (2 h)



Heatmap 20 ppm (2 h)



Heatmap 10 ppm (24 h)

Figure 2 Continued.

https://doi.org/10.2147/IDR.S498943

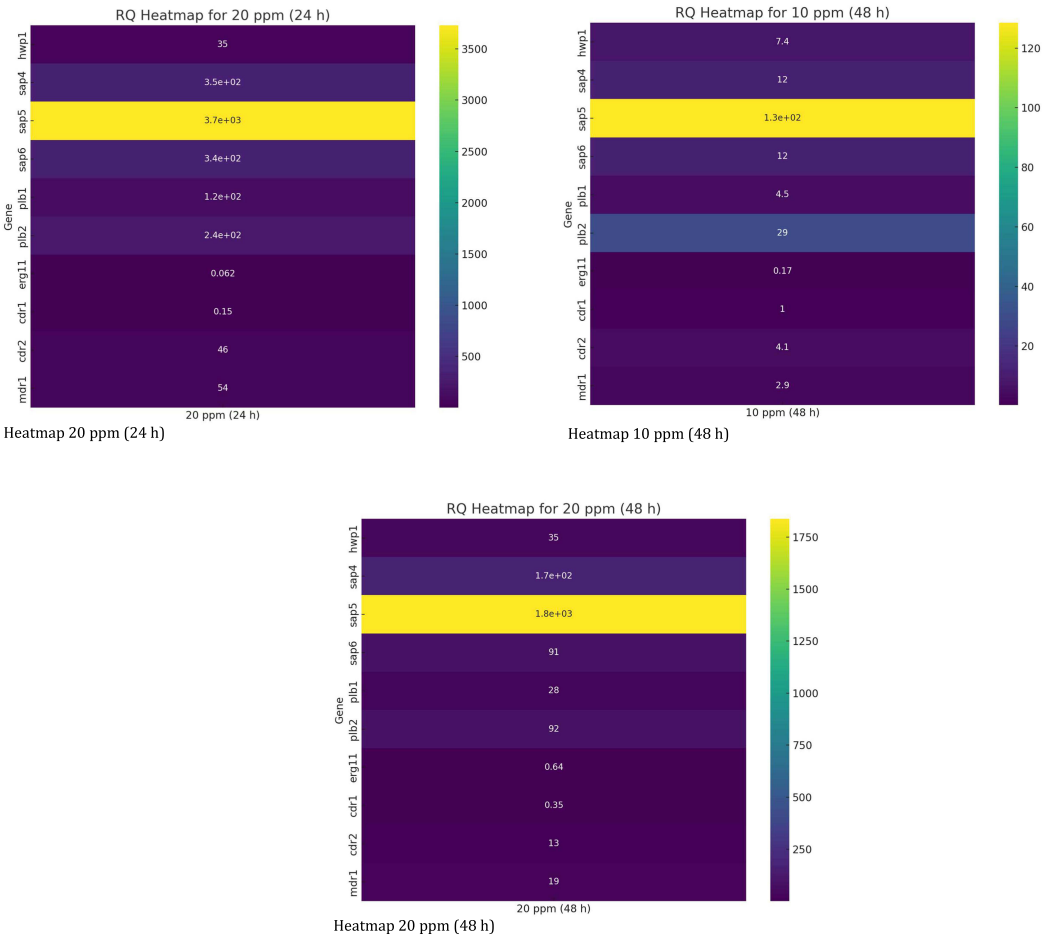


Figure 2 Heatmaps of Gene expressions at HEX concentrations of *C. albicans* incubated at 0, 10 and 20 ppm for 0.5, 2, 24, and 48 h.

low concentrations of antiseptics influence drug resistance and the pathogenicity of *C. albicans* during treatment. The effects of sub-therapeutic concentrations of oral antiseptics on *C. albicans* virulence factors have been examined phenotypically in many studies.[10,13,37] To our knowledge, this is the first study to investigate the impact of oral antiseptic hexetidine (HEX) on the expression levels of genes associated with virulence and drug resistance. Only one previous study examined the effect of cetylpyridinium chloride (CPC) on the expression of the *cdr1* and *cdr2* genes, which are involved in drug resistance, and reported the overexpression of both genes;[38] however, this study did not assess virulence gene expression.

The aim of our study was to determine the effect of HEX on the expression of virulence factor genes (*hwp1, sap4, sap5, sap6, plb1*, and *plb2*) and drug resistance genes (*mdr1, erg11, cdr1*, and *cdr2*). Oral antiseptics are known to be quickly absorbed by *C. albicans*, within 15–30 seconds.[39] This study examined the effects of both short-term (30 minutes and 2 hours) and long-term (24 and 48 hours) exposure to the antiseptic on the expression of *C. albicans* virulence and drug resistance genes.

Virulence factors, including adhesion, biofilm formation, and hydrolytic enzyme activity, play a critical role in the pathogenesis of *Candida albicans* infections. The primary aim of this study was to determine how sub-therapeutic doses of HEX influence the expression of virulence-associated genes, such as *hwp1, sap4, and plb1*.

Although resistance genes such as *cdr1* and *erg11* were analyzed to provide a broader understanding of the molecular impact of HEX, these findings were secondary to the primary aim of understanding virulence regulation.

In this study, the effects of antiseptics at low doses and varying exposure durations on *C. albicans* were examined in detail. The findings demonstrate that HEX induces significant changes, particularly in virulence and resistance genes such

as *hwp1, sap4, sap5*, and *mdr1*. The expression of the *hwp1* gene was observed to decrease during both short-term (0.5 hours) and long-term (24 and 48 hours) exposure. This gene plays a critical role in *Candida*'s adhesion to mucosal surfaces and is highly expressed during the early stages of biofilm formation. The suppression of this gene's expression by HEX may impact biofilm formation and, consequently, the initial stages of infection.

Additionally, with regard to the *sap* gene family (*sap4, sap5*, and *sap6*), HEX exposure increased the expression levels of these genes. *Sap* proteinases play a central role in the degradation of host cell membranes and tissue invasion. The upregulation of these genes at low doses of HEX suggests the need for further studies to better understand the impact of antiseptics on host-pathogen interactions.

Among resistance genes, the expression of *erg11* was found to decrease following 0.5-hour and 24-hour exposure to HEX. The *erg11* gene plays a significant role in the resistance mechanism to azole antifungals, and its reduced expression suggests increased antifungal susceptibility. However, the *cdr1* and *cdr2* genes exhibited a significant decrease in expression during prolonged exposure to low doses of HEX. These genes encode ABC transporter proteins that mediate the efflux of antifungal drugs from the cell, and their overexpression is associated with the development of resistance.

The findings demonstrate that HEX significantly modulates virulence gene expression, particularly those involved in adhesion and biofilm formation. These results emphasize the importance of targeting virulence factors as a therapeutic strategy against *Candida* infections.

These findings demonstrate that the antiseptic had varying effects on gene expression depending on the duration of exposure. While the discussion could not fully explore some of the intriguing correlations due to the lack of similar studies involving HEX and *Candida albicans*, these results will likely contribute to the development of new treatment approaches in the future. The study's evaluation period of 48 hours exceeds the typical timeframes examined in other studies and is clinically relevant, as patients generally brush their teeth at least once daily and sterilize their dentures. These findings provide potential alternatives for the treatment of oral candidal infections.

To further advance these findings toward clinical application, additional analyses such as drug release profiles, cytotoxicity studies, and biofilm assessments are necessary to support the transition to clinical trials as an alternative therapy for oral candidal infections.

The potential of essential oils (EOs) has been highlighted as a promising avenue for discovering new classes of antifungals. Many studies have investigated the antifungal properties of EOs and their potential applications in fungal control, calling for further discussion.[40–42] Additionally, combination therapy presents a cost-effective alternative, as it bypasses the expensive and time-consuming process of developing new drugs.[43]

The findings of this study demonstrate that antiseptics can influence virulence and resistance gene expression during prolonged exposure at low concentrations. Specifically, HEX was shown to significantly modulate the expression of virulence genes involved in adhesion and biofilm formation. These results emphasize the importance of targeting virulence factors as a therapeutic strategy against *Candida* infections.

This study contributes to the literature as one of the first to examine the effects of HEX on the virulence gene expression of *C. albicans*. However, further research is required to translate these findings into clinical applications. In particular, the effects of HEX on biofilm inhibition should be detailed with various dose and duration combinations. Additionally, collaboration with biomedical engineers could facilitate the development of drug delivery systems that allow antiseptics to still be longer in the oral cavity. Such systems could mitigate the effects of saliva washing and mechanical cleansing, enhancing the retention and efficacy of the antiseptic.

Conclusion

This study highlights the potential of HEX as a modulator of virulence gene expression in *Candida albicans*. The significant downregulation of key virulence genes such as *hwp1* and *plb1* suggests that subtherapeutic doses of HEX may serve as an adjunct therapy for *Candida* infections. The uniqueness and clinical significance of this research lie in its investigation of how low doses of HEX affect gene expression over various periods, offering potential insights into alternative therapeutic approaches.

Future studies should focus on optimizing HEX concentrations to maximize its impact on virulence without promoting resistance.

Data Sharing Statement

Data used to support the findings of this study are available from the corresponding author upon request.

Funding

This work was supported by Marmara University Scientific Research Projects Committee (No. SAG- B-040712 0261).

Disclosure

The authors report no conflicts of interest in this work.

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