The inhibitory effects of tyrosol on clinical *Candida glabrata* planktonic and biofilm cells

Research Article

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ABSTRACT

Biofilm formation is an important problem in the healthcare industry and veterinary medicine and is very common in natural, industrial or hospital environments. Microorganisms can become very resistant to antimicrobials and environmental factors by biofilm forming on biotic or abiotic surfaces. There is a need to develop new, effective and specific antimicrobials that can reduce pathogenicity in biofilm formation that threatens public health due to their role in medical device-related or infectious diseases. *Candida* species are opportunistic pathogenic yeasts and can cause superficial or disseminated infections. Especially *C. glabrata* is one of the most common microorganisms causing fungal infections in immunocompromised patients and drug resistance is observed when associated with biofilm. Tyrosol (2-[4-hydroxyphenyl] ethanol) can act as both a quorum sensing molecule and an exogenous agent on *Candida* species. In this study, the antifungal activity of tyrosol against a clinical *C. glabrata* isolate was investigated on both planktonic and biofilm forms. Broth microdilution test results demonstrated the inhibitory effect of tyrosol on *C. glabrata*. Transmission electron microscopic findings showed that tyrosol affected the planktonic *C. glabrata* cells in a multi targeted manner, and in the groups treated with tyrosol, significant damage was observed in the cell wall, cell membrane, cytoplasm, nucleus and mitochondria. Also, scanning electron microscopic images confirmed biofilm reduction in pre-/post-biofilm applications as a result of tyrosol treatment. In conclusion, tyrosol may be a potential alternative candidate for reducing the C. *glabrata* biofilm.

Keywords: Biofilm, *C. glabrata*, Tyrosol, Electron Microscopy.

INTRODUCTION

Microorganisms form biofilms on host tissues, domestic/industrial surfaces, biomaterials such as prostheses and catheters, and can cause highly resistant infections relative to their planktonic form (Lison et al., 2022; Sharma et al., 2019). The robust architecture of the biofilm contributes to resistance by both reduced metabolic activity and the presence of the extracellular matrix. There are several steps in the formation of a typical microbial biofilm, such as attachment to living or non-living surfaces, maturation and dispersion. Therefore, biofilms can act as a reservoir for pathogenic cells, and their release can cause septicemia, leading to disseminated systemic infections of organs and tissues. It is reported in the literature that approximately 80% of microbial infections are associated with biofilms and show high mortality rates (Srinivasan et al., 2017; Chen and Wen, 2011). Thus, there is an urgent need for new anti-biofilm approaches to control and eradicate these infections.

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It is stated in the literature that most of the devices with medical applications can cause biofilm infections. *Candida* is prominent in biofilms formed by clinically important fungi. Especially *C. albicans* can form a biofilm in almost every medical device and can cause infection by passing into the bloodstream from where it is located. Although *C. albicans* is the most frequently isolated *Candida* species, there has been a significant increase in the frequency of non-*albicans* species such as *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. krusei* in recent years due to various reasons such as immunosuppression, various diseases, prematurity, exposure to broad-spectrum antibiotics. (Ramage et al., 2005).

One of the interesting and effective methods proposed in the biofilm management in recent years is the use of Quorum sensing (QS) molecules. When the cell density in the biofilm reaches a certain level, microorganisms secrete QS molecules and various physiological activities are regulated by the modification of target genes. Tyrosol is one of the major QS molecules isolated from *C. albicans*. Aromatic alcohol tyrosol is known to induce yeast-hyphae transition, but studies on its antibiofilm activity are very limited. However, it is reported that tyrosol has a biofilm-reducing effect when used in combination with some known antifungals (Arias et al., 2016). On the other hand, exogenously applied tyrosol stimulates hyphae production in the early stages and before some cells start hyphal development (1-6 hours). This condition shows that tyrosol acts as a QS molecule on both planktonic cells and biofilm (Rodriguez and Cernakova, 2020). However, information on the antibiofilm effects of tyrosol on *C. glabrata* is very limited in the literature.

To study fungal biofilms, improvable *in vitro* models are needed. The effects of various inhibitory substances on these models can be studied at different concentrations or the effects of different growth environments can be investigated. Especially, the oral cavity, which contains thousands of bacterial and nearly a hundred fungal species, attracts attention as a valuable research area for biofilm models. In this study, it was aimed to investigate the effectiveness of *C. glabrata* on planktonic and biofilm forms as a result of exogenous application of tyrosol, a newly defined QS molecule. In addition, acrylic resin was used to mimic the oral biofilm with an *in vitro* model. Microbiological and electron microscopic techniques were used in the study.

MATERIAL and METHOD

Preparation of yeast suspension

The clinical *C. glabrata* 1744 isolate used in the study was obtained from Eskisehir Osmangazi University, Faculty of Medicine, Department of Microbiology and was selected among the strains with strong biofilm characteristics. This isolate was maintained on glycerol/SDB (20/80 v/v) at −80 °C in Eskisehir Osmangazi University Central Research Laboratory, Application and Research Center (ARUM), Biotechnology Laboratory. *C. glabrata* 1744 isolate from the stock culture was activated in YPD (Yeast Extract Peptone Dextrose) medium at 37 °C, and then incubated in RPMI 1640 broth and 37 °C for 24 hours. The sample was then taken up in 5 ml of 0.85% saline. The turbidity of the suspension was adjusted to 0.5 McFarland (1-5 x 10^6 cells/ml). The prepared initial suspensions were diluted 1/50 with sterile saline and then 1/20 with RPMI 1640, and a concentration of 1-5 x 10^3 cells/ml was obtained.

Susceptibility of planktonic C. glabrata to tyrosol

The determination of the Minimum Inhibition Concentration (MIC) of tyrosol ((2-[4-hydroxyphenyl]) (Sigma Chemical Co., USA)) was based on the Clinical and Laboratory Standards Institute Microdilution Reference

Method (CLSI M27-A3). Briefly, cell suspensions of isolates were adjusted to the 0.5 McFarland standard in saline solution and diluted in Roswell Park Memorial Institute (RPMI 1640, Sigma Aldrich) medium. Tyrosol was first diluted in deionized water and then diluted in RPMI 1640 medium to obtain concentrations ranging from 600-1.17 µg/ml. Then, 100 µl of each cell suspension and 100 µl of each tyrosol concentration were pipetted into 96-well microplates. Plates were incubated at 37 °C for 48 hours and MIC value was defined as the lowest drug concentration that inhibited the growth of microorganisms. Standard control antifungal Amphotericin B (Sigma, USA) (AppliChem A1907,0050) was dissolved in DMSO (dimethyl sulfoxide) and its final concentrations were adjusted to be 16-0.0313 µg/ml. After the microdilution process was carried out as stated above and the MIC of amphotericin B was defined as the lowest drug concentration that complete inhibition of visible growth (CLSI M27-A3). All the experiments were performed in triplicate and results were averaged (Dağ et al., 2018).

Transmission electron microscopy (TEM)

In the study, TEM was used to reveal the ultrastructural effects of tyrosol on planktonic *C. glabrata*. The medium and tyrosol solutions prepared at MIC and ½ MIC concentrations were added to sterile 6-well tissue culture plates. Microorganism suspensions were also inoculated at 200 µl per well. A control group without tyrosol was also prepared on the same plate, and absorbance measurements at 490-600 nm were made after 24 hours of incubation at 37°C.

For the routine TEM procedure of the samples, the cell suspensions taken into sterile centrifuge tubes were centrifuged for 15 minutes, the supernatant was removed, and the obtained pellet was washed with PBS buffer 3 times for 10 minutes and taken into 2.5% glutaraldehyde (in PBS) medium for primary fixation (24 hours at +4 °C). After washing with PBS 3 times for 15 minutes, the samples were left in osmium tetraoxide in the dark for 1 hour with the help of a rotator, and then washed again with PBS 3 times at the end of the period. Samples embedded within 1.5 % agar were dehydrated in a series of ethanol at increasing concentrations after block staining with 1% uranyl acetate. After clarification with propylene oxide and embedding in resin, the samples were polymerized in an incubator at 60 °C for 48 hours, 60 nm thick sections were taken on copper grids with the help of an ultramicrotome (Leica UltraCutR) and stained with uranyl acetate+lead citrate. Then samples were analyzed with TEM device (Hitachi HT 7800), (Yapıcı et al., 2021; Öztürk et al., 2020).

Scanning electron microscopy (SEM)

Prebiofilm study

The effect of tyrosol on biofilm formation was tested by SEM and the biofilms were developed on the surface of the acrylic resin support materials (Acrystone, 8 x 4 mm) placed in 24-well plates. Acrylic resin samples were sterilized under UV before the study. To study the prebiofilm effects, tyrosol were applied to the cells at the beginning (0 h) of the study and at the three different ranges. For this purpose, acyrilic resin pieces were replaced in 24 well plates. Tyrosol was prepared in RPMI 1640 at MIC concentration and dilutions were added to each well. The turbidity of the yeast suspension was adjusted to 0.5 Mc Farland. Each well of 24 well plates was inoculated with standardized cell cells. Inoculated RPMI 1640 without tyrosol was also included to study. Amphotericin B was used as control antifungal. Chlorhexidine gluconate (Pharmactive, %0,12) was also used as positive control. Then the samples were taken into the routine SEM procedure (Yapici et al., 2021).

Postbiofilm study

The effectiveness of tyrosol on the formed biofilm was used and analyzed by SEM device,

as it could give an idea about the therapeutic properties of tyrosol. For this purpose, biofilms were developed by inoculating standardized cell suspensions developed in RPMI 1640 medium on acrylic resin surfaces placed in 24-well tissue culture plates, and then incubated for an additional 24 hours by adding MIC concentration of tyrosol. The samples were then subjected to the routine SEM procedure (Yapıcı et al., 2021)

SEM analysis

After the samples were incubated in 2.5% glutaraldehyde (in PBS) for 24 hours at +4 °C, they were washed 3 times with PBS for 15 minutes to remove the fixative. The samples, which were taken into osmium tetroxide for secondary fixation, were kept in the dark for 1 hour with the help of a rotator and were washed 3 times with PBS again. After the samples were dehydrated with increasing concentrations of ethanol, they were were dried on aluminum stubs, coated with gold-palladium (Polaron SC7620 Sputter Coater) and analysed with SEM device (HITACHI Regulus 8230), (Öztürk et al., 2022; Seneviratne et al., 2009).

RESULTS

According to the results of *in vitro* antifungal susceptibility test results of *C. glabrata* 1744 isolate, MIC value of tyrosol was found as 18.75 µg/ml. The MIC value of Amphotericin B, which is widely used in the treatment of fungal diseases, was found to be lower than tryosol (0.78 µg/ml).

The data obtained by TEM revealed the ultrastructural changes in the tyrosol treated groups compared to the control group. Untreated control *C. glabrata* cells exhibited typical and healthy *Candida* morphology. The nucleus is prominent and centrally located, the cytoplasm is regular, the cell wall and cytoplasmic membrane structure were observed as a whole. Mitochondria and cristae structures were also clearly observed (Figure 1A-C). In cells exposed to tyrosol at ½ MIC concentration, a marked enlargement between the wall and the membrane was remarkable. Also, deformations in the cells, occasional cytoplasmic ruptures, severe nuclear damage, cytoplasmic lysis, structural disruptions in the cell membrane, and a small number of ghost cells were detected (Figure 1D-F). Cells exposed to MIC concentration of tyrosol similarly showed cytoplasmic lysis, cell wall and membrane damage, nuclear damage, and mitochondrial greying (Figure 1G-I).

In this study, the effects of tryosol on *C. glabrata* biofilm were investigated by SEM. In the control group samples, smooth-surfaced and healthy-appearance biofilm cells were found on the acrylic surface (Figure 2A). When the cells were exposed to tyrosol at MIC concentration before the biofilm formation (prebiofilm study), some bleb formations and cell shrinkages were observed (Figure 2B). Cells were exposed to tyrosol at MIC concentration after the biofilm formation (postbiofilm study), cellular deformities appeared in many different ways. Damages in the form of indentations, fused cells or shape deformities were determined (Figure 2C). In the samples treated with antifungal AMB at MIC concentration, a significant decrease was observed in the cells compared to the control, but it was determined that the cell morphologies were largely preserved (Figure 2D). In the chlorhexidine-administered groups, the number of cells decreased compared to the control, and these few cells were also damaged (Figure 2E).

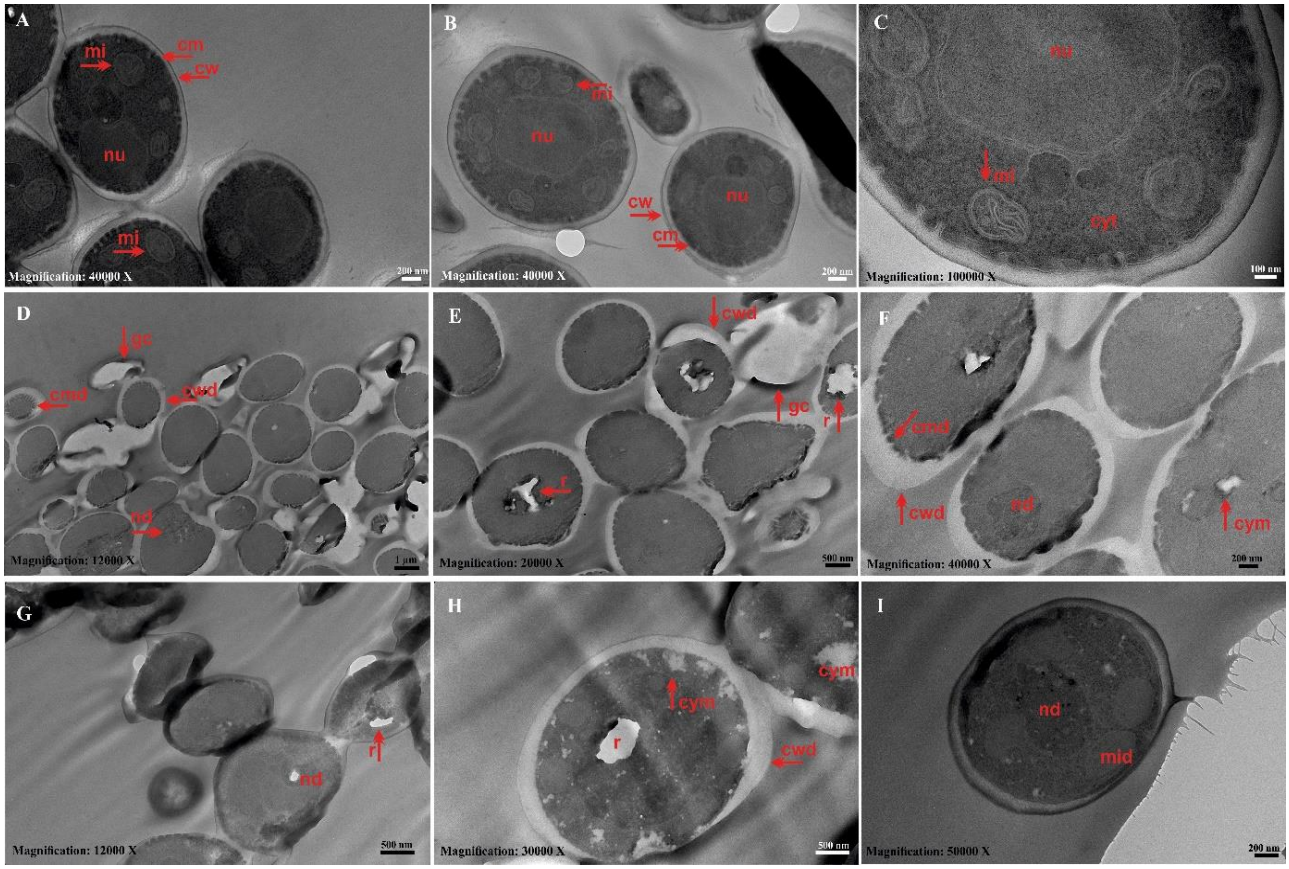

Figure 1. Demonstrative TEM images of *C. glabrata* 1744 cells before and after exposure to tyrosol, showing ultrastructural changes. (A–C) Untreated control cells revealed normal cell morphology with centrally located nucleus, regular cytoplasm, intact cell wall and cytoplasmic membrane structures and healthy mitochondria (A and B= 200 nm, C = 100 nm scales); (D-I) Cells treated with tyrosol at subinhibitor concentration (1/2 MIC) showed some irregularities and damages with marked enlargement between the wall and the membrane structure, membrane deformations, cytoplasmic ruptures, severe nuclear damage, cytoplasmic lysis and ghost cells (D= 1µm, E= 500 nm, F = 200 nm scales); (G-I) Cells treated with tyrosol at subinhibitor concentration MIC) exhibited cytoplasmic lysis, cell wall and membrane damage, nuclear damage, and mitochondrial greying (G and H= 500 nm, I = 200 nm scales) Abbreviations: mi mitochondria, nu nucleus, cvm cytoplasmic meltings, nd nuclear damage, md membrane damage, mid mitochondrial damage, r ruptures, gc ghost cell, cwd cell wall damage

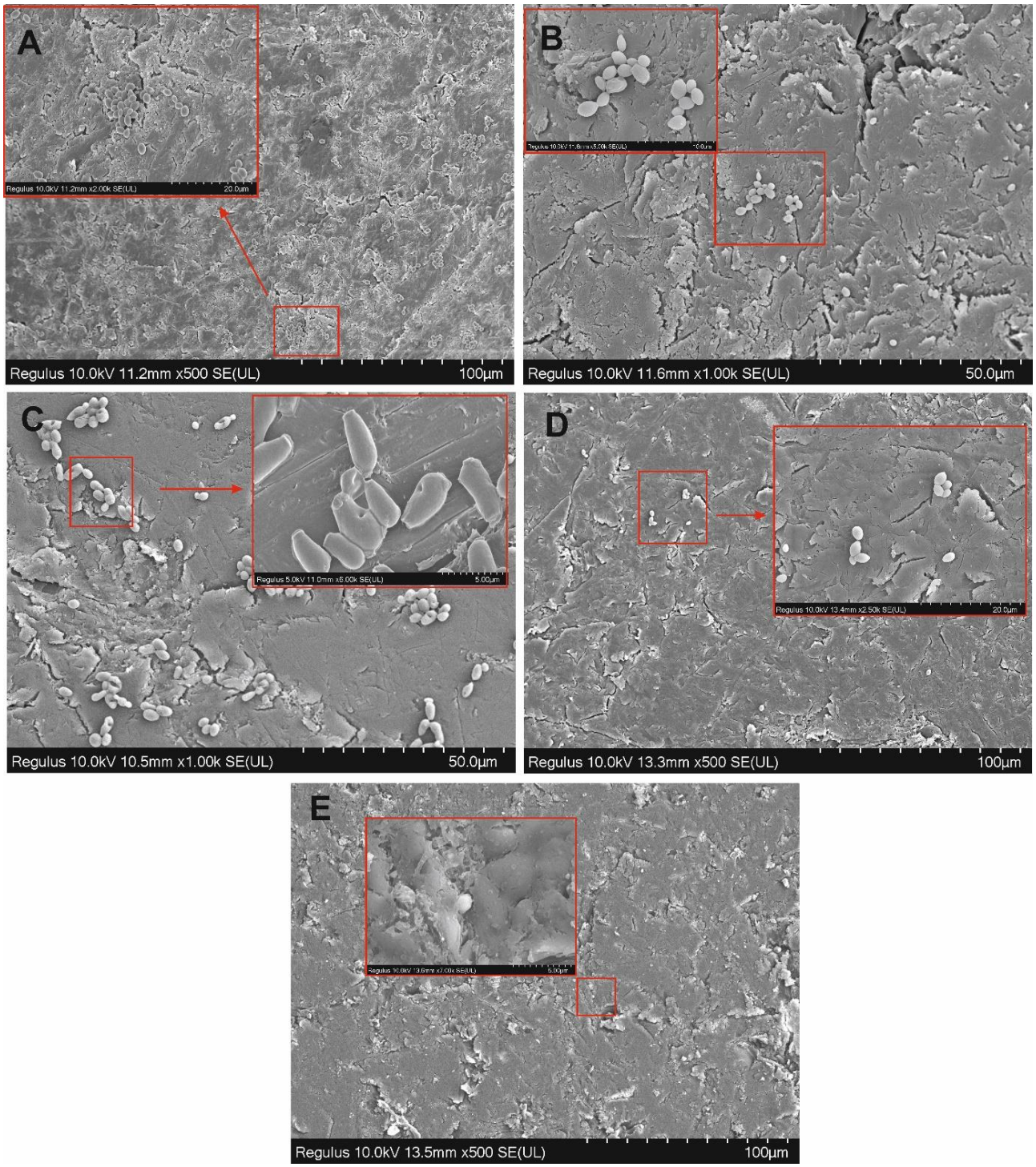

Figure 2. Demonstrative SEM images of *C. glabrata* 1744 biofilm with and without tyrosol on acyrlic resin surfaces, showing morphological changes. (A) Untreated control group biofilm dense biofilm and EPS structure (A= 100 µm and larger magnification of same image at the upper left corner 20 µm); (B) Cells treated with tyrosol at MIC concentration before the biofilm formation (prebiofilm study) revealed some bleb formations and cell shrinkages (B= 50 µm and larger magnification of same image at the upper left corner 10 µm); (C) Cells treated with tyrosol at MIC concentration after the biofilm formation (postbiofilm study), cellular deformities with indentations, fused cells or shape deformities (C= 50 µm and larger magnification of same image at the upper right corner 5 µm); (D) Cells treated with amphotericin B at MIC concentration showed significant decrease in biofilm cells but intact cell structures (D= 100 µm and larger magnification of same image at the upper right corner 20 µm); (E) Cells treated with chlorhexidine gluconate showed decreased biofilm and few damaged cells (E= 100 µm and larger magnification of same image at the upper left corner 5 µm).

DISCUSSION

Biofilm formation is a mechanism that allows many microorganisms to survive in the host environment and plays a very important role in antimicrobial resistance. Biofilms, also called microbial communities that adhere to surfaces, organize under the control of QS signaling molecules (Zhou et al., 2020). This event is a communication mechanism by synchronized gene expression in response to the cell density population (Sharma et al., 2019). Biofilm-associated infections, which can develop on the surface of biomaterials such as indwelling devices and in various tissues, are considered an important and increasingly common source of morbidity and mortality in the healthcare system. Biofilm eradication is difficult and often requires removal of the infected indwelling device, and biofilm is the main source of many persistent infections. Therefore, much more research is needed in terms of biofilm prevention and management.

The QS event in fungi is relatively new and only started to be understood after the discovery of farnesol, which discovery the filamentation process in *C. albicans*. After this discovery, tyrosol was also described as a QS molecule in *C. albicans*, and its roles on growth, morphogenesis and biofilm formation began to be elucidated. In *Candida*, farnesol induces the transition from yeast to hyphae and biofilm formation, while tyrosol has the opposite effect. On the other hand, such QS molecules can also be applied exogenously and act as an antimicrobial agent against various fungal and bacterial pathogens (Monteiro et al., 2017). Currently, fungal QS research is in initial level and studies on the effects of tyrosol on non-albicans *Candida* are very limited (Rodriguez and Cernakova, 2020). In this study, the *in vitro* inhibitory effects of tyrosol on a clinical *C. glabrata* isolate were investigated.

In the literature, it is seen that the MIC values obtained for QS molecules such as tyrosol and farnesol against microorganisms such as *Candida* and *Streptococcus mutans* show differences (40 µmol l^{-1} - 5 mmol l^{-1}). Cordeiro et al. (2015) investigated the effect of high concentrations of exogenous tyrosol on planktonic cells and biofilms of *C. albicans* (n = 10) and *C. tropicalis* (n = 10) isolates, and evaluated whether tyrosol was synergistic with antifungals targeting cellular ergosterol. The authors studied concentration ranges of 0.04 - 22 mM in the study, which is approximately 2x - 1000x times the concentration reached endogenously in *C. albicans*. According to the MIC data obtained as a result of broth microdilution, planktonic cells exhibited varying MIC values of 2.5 - 5.0 mM. In addition, a high rate of synergy was found in combinations of tyrosol with AMB, itraconazole and fluconazole. In another study by Monteiro et al. (2015), the effect of tyrosol on the adhesion of *C. albicans* and *C. glabrata* on acrylic surfaces was investigated. The authors reported that *C. albicans* isolate was more sensitive to tyrosol than *C. glabrata*. While the MIC value was 50 mM and the MFC value was 90 mM for *C. albicans*, the MIC value was 90 mM and the MFC value was 100 mM for *C. glabrata*.

In another study, the effectiveness of tyrosol on single and mixed biofilms of *C. albicans*, *C. glabrata* and *S. mutans* on acrylic resin and hydroxyapatite surfaces was investigated. The MIC values obtained for tyrosol in the study were found to be higher than these values reported in the literature (MIC for *C. glabrata*: 90 mmol l^{-1}, MFC: 100 mmol l^{-1}). The authors stated that the different MIC results in these studies can be explained by the different susceptibility profile in various strains. In the same study, tyrosol, especially used at a concentration of 200 mmol l^{-1}, led to a significant decrease in the number of CFUs and metabolic activity for all single and mixed biofilms formed on both surfaces. Cell damage after tyrosol exposure was also supported by SEM examinations (Arias et al., 2016). In our

study, the MIC value obtained for tyrosol was 18.75 µg/ml. This value is generally lower than the above-mentioned study data. However, as stated in the literature, MIC data show a wide range in studies related to tyrosol, and the differences may vary depending on the strain. Also, one of the limitations of our study is that we only studied with a single isolate, so it seems that there is a need for detailed studies on the subject.

When the effects of MIC and ½ MIC concentration of tyrosol exposure on planktonic *C. glabrata* cells were analyzed by TEM, it was determined that tyrosol had a multi-targeted effect. To our knowledge, there is no study in the literature examining the effects of tyrosol on *C. glabrata* at the ultrastructural level, but there are some studies examining the effects of farnesol on *Candida* species by TEM. In a previous study, the antibiofilm effects of farnesol on *Candida* species were investigated, and cell wall-membrane separations, ghost cell formations, cytoplasm damage, vacuol formation, some completely lyzed cells were detected in the TEM data obtained as a result of exposure to 37.5 µM farnesol on *C. albicans* ATCC 14053 (Yapıcı et al., 2021). In another study, a relatively regular morphology was observed in *C. albicans* cells treated with farnesol, and membrane and wall damage and electron density were observed in a small number of cells (Yenice Gürsu, 2020). Decanis et al (2011) also reported large and irregular cytoplasmic vacuoles and especially peripheral vacuoles regarding the ultrastructural effects of farnesol on *C. albicans*, and showed cell wall and membrane damage and cytoplasmic granulation findings. However, in our study, the effects of tyrosol on *C. glabrata* were significantly damaging and significant damage was observed at the level of cell wall, membrane, nucleus and mitochondria.

According to the data we obtained with SEM, it was determined that there were biofilm reduction and cellular damage as a result of both prebiofilm and post biofilm applications after tyrosol exposure. In a previous study, the effects of a combination of tyrosol and farnesol were investigated against *C. albicans* and *C. glabrata* isolates in planktonic or biofilm form. In the study, total biomass, metabolic activity and the amount of cultivable cells were determined; tyrosol and farnesol showed a synergistic effect against *C. glabrata* planktonic cells. In addition, in studies in which both single and dual biofilms were created, there was a significant reduction in total biomass and metabolic activity for *C. glabrata* biofilms, and the results were confirmed by SEM studies. The biofilm structure of *C. glabrata* was thinner and partially covered the surface in groups where the active substance was applied alone or in combination (Monteiro et al., 2017). In also another study, the effects of tyrosol and chlorhexidine gluconate in combination against planktonic or biofilm cells of *C. albicans*, *C. glabrata* and *S. mutans* were investigated. The authors classified the effect of the drug combination on planktonic cells as antagonistic for *C. albicans* and as indifferent for all other strains. The drugs showed indifferent effects on the tested biofilms. However, the drug combination showed a synergistic effect in reducing the number of *C. albicans* hyphae (Do Vale et al., 2017). In also our study, the reducing effects of tyrosol on *C. glabrata* biofilm and damaging cells were observed. It has been reported in the literature that tyrosol can act by inhibiting the incipient production of the extracellular matrix. (Monteiro et al., 2015; Li et al., 2007). Although a strong decrease in biofilm was observed in the groups treated with AMB and chlorhexidine compared to the control, it was determined that the morphology was better preserved in a small number of cells on the surface. It is known that chlorhexidine has important effects in the fight against biofilm-related oral infections, and it is used as the main component in toothpastes,

mouthwashes or gels. In our study, strong effects of chlorhexidine on the *C. glabrata* biofilm on the acrylic surface were observed. The EPS structure observed in the biofilm structure could not be seen clearly in the SEM images in our study, but it is thought that the dehydration processes applied during electron microscopic follow-up reduce EPS. In addition, the curved nature of acrylic resin created some difficulties in SEM examinations, such as charging or loss of clarity in some areas.

C. albicans is a polymorphic yeast that can form pseudohyphae and true hyphae. *C. glabrata* is a species that grows only as yeast, and the MIC and MFC values obtained for this species are higher than *C. albicans*. Although *C. albicans* is the main pathogen in cases of denture stomatitis, the lower susceptibility of *C. glabrata* proves that this species is resistant to conventional antifungals, and this issue stands out as an important health problem (Meşeli et al., 2019; Hannah et al., 2017). While the microbiota in the oral cavity protects the human body against infectious diseases, various oral infections may develop due to reasons such as immunodeficiency, malnutrition, poor oral hygiene or the use of improperly fitting removable dentures. Denute stomatitis is a chronic oral fungal infection that significantly affects denture wearers. This infection has often been associated with microbial biofilms on the acrylic base and prosthesis-bearing mucosa. Removable acrylic resin prostheses provide adequate physical, mechanical and aesthetic properties, but the resinous nature of these materials makes them vulnerable. Local factors such as mismatched dentures, rough or cracked denture surfaces, weak saliva, mucous discharge, or proliferation of *Candida* species on the denture surface can lead to the development of denture stomatitis (Bajunaid et al., 2022., Monteiro et al., 2015; Bianchi et al., 2016). In our study, acrylic resin tooth samples were used to imitate the oral biofilm model and it was observed that tryrosol had reducing effects on *C. glabrata* biofilms growing on the surfaces.

CONCLUSION

In conclusion, investigating the effects of QS molecules as one of the new antifungal strategies targeting biofilm formation and growth in *Candida* is a promising topic. The obtained data can give an idea about prophylactic approaches, especially in providing oral hygiene. Tyrosol showed a significant inhibition effect on both planktonic and biofilm forms of *C. glabrata*. On the other hand, since the problem of antimicrobial resistance to existing antifungals is a very important health problem, the combined use of new antifungal candidate agents such as tyrosol with antifungals may produce stronger effects. However, it is necessary to verify the data by making detailed studies on this subject.

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Ethical approval:

We declare as the authors of the study that the approval of the Ethics committee is not required within the scope of the presented study.

Conflict of interest: The authors declare that there is no conflict of interest for this study.

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