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RESEARCH ARTICLE

Antibiofilm Potential of Ceragenins Against Vancomycin-Resistant *Enterococcus* spp.

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SUMMARY

Enterococcus spp., especially vancomycin-resistant enterococci (VRE), are listed by the World Health Organization as priority pathogens requiring new antimicrobial treatments. Ceragenins (CSAs) are a novel class of broad-spectrum antimicrobials that are relatively easy and cost-effective to produce. This study evaluated the antibiofilm effects, anti quorum-sensing (QS), and antioxidant capacities of ceragenins (CSA-44, CSA-90, CSA-192) and linezolid against VRE isolates from various clinical samples. CSA-44 and CSA-192 most effectively inhibited initial adhesion and biofilm formation. For mature biofilms, CSA-90 showed the greatest inhibition (~66%) while CSA-44 and CSA-192 reached ~50%. Linezolid inhibited biofilms by 39–60%, depending on concentration. No anti-QS or antioxidant activity was observed for ceragenins at the tested levels. These findings suggest ceragenins have strong potential as therapeutic agents against VRE, particularly in biofilm-related infections. Further in vivo research is needed to confirm their efficacy and safety for clinical use.

Keywords: *Biofilm, cationic antimicrobial peptides, Enterococcus, linezolid, vancomycin resistance.*

Vankomisine Dirençli Enterokok Türlerine Karşı Cerageninlerin Antibiyofilm Etkinliği

ÖZ

Enterococcus spp., özellikle vankomisine dirençli enterokoklar (VRE), Dünya Sağlık Örgütü tarafından yeni antimikrobiyal tedaviler gerektiren öncelikli patojenler olarak belirlenmiştir. Cerageninler (CSA'lar), üretimi nispeten kolay ve uygun maliyetli yeni bir geniş spektrumlu antimikrobiyal sınıfıdır. Bu çalışmada, çeşitli klinik örneklerden izole edilen VRE'lere karşı cerageninlerin (CSA-44, CSA-90, CSA-192) ve linezolidin antibiyofilm, anti quorum-sensing (QS) ve antioksidan etkileri değerlendirilmiştir. CSA-44 ve CSA-192, biyofilmi yapışma ve oluşum aşamasında en etkili şekilde inhibe etmişlerdir. Olgun biyofilmler için CSA-90 en yüksek inhibisyon oranını (~%66) gösterirken, CSA-44 ve CSA-192 ~%50'ye varan oranlarda inhibisyon göstermiştir. Linezolid, konsantrasyona bağlı olarak biyofilmleri %39-60 oranında inhibe etmiştir. Çalışılan konsantrasyonlarda cerageninler için herhangi bir anti-QS veya antioksidan aktivite gözlemlenmemiştir. Bu bulgular, cerageninlerin özellikle biyofilm ile ilişkili enfeksiyonlarda VRE'ye karşı terapötik ajanlar olarak güçlü bir potansiyele sahip olduğunu göstermektedir. Klinik kullanım için etkinlik ve güvenilirliklerini doğrulamak amacıyla daha fazla in vivo araştırma yapılması gerekmektedir.

Anahtar Kelimeler: *Biyofilm, katyonik antimikrobiyal peptitler, Enterococcus, linezolid, vankomisin direnci.*

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INTRODUCTION

Enterococcus spp. are Gram-positive bacteria found in soil, surface water, seawater, plants, and fermented foods. *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) are the dominant species in this genus. This facultative anaerobic genus is highly tolerant to a wide range of environmental conditions, including extremes of pH, temperature, and salt concentration. This tolerance contributes to their colonisation and persistence in various environments. They are primarily commensals in the gastrointestinal tract but can cause a range of infections. The main infections they cause include bacteremia, endocarditis, and urinary tract infections. In addition to their potential to cause infections, enterococci are also characterised by their resistance to many antibiotics. They show both intrinsic and acquired resistance to many antimicrobials used to treat Gram-positive bacterial infections, particularly vancomycin (Agudelo Higuita & Huycke, 2014; Ch'ng et al., 2019; Garcia-Solache & Rice, 2019).

In 2019 and 2021, 1.27 and 1.14 million deaths, respectively, were known to be directly related to antibiotic-resistant bacteria, and 4.71-4.95 million deaths annually were indirectly related to bacterial antimicrobial resistance. Due to limited treatment options, bacteraemia caused by vancomycin-resistant enterococci (VRE) leads to higher morbidity and mortality than bacteraemia caused by vancomycin-sensitive enterococci. In 2019, *E. faecium* and *E. faecalis* were responsible for 100,000 to 250,000 of these deaths, with vancomycin-resistant strains accounting for a high percentage (Humphreys, 2014; Naghavi et al., 2024). VRE has been recognized as a global public health problem by the World Health Organization (WHO), US Centers for Disease Control and Prevention (CDC), and European Centre for Disease Prevention and Control (ECDC). In recent years, the epidemiology of VRE has changed rapidly and markedly, and an increase in vancomycin resistance, particularly in *E. faecium*, has been observed in many countries. Linezolid, daptomycin and tigecycline are antibiotics of last resort in the treatment of VRE infections. However, resistance to these antibiotics has also been reported (CDC, 2019; Dadashi et al., 2021; ECDC, 2023; Tacconelli et al., 2018).

It is a global health priority to combat antibiotic resistance in enterococci. Although *E. faecalis* forms thicker biofilms that confer resistance to antibiotics, *E. faecium* is intrinsically more resistant to antibiotics, especially in biofilm form. The underlying mechanism for the emergence of antibiotic resistance to biofilms has not yet been determined, but it may be due to high levels of matrix production in mature biofilms and thus low penetration of antibiotics, or to an increase in antibiotic-tolerant cells, as in other bacterial species (Komiyama et al., 2016; Soares et al., 2014). Biofilms are loosely defined as clusters of bacteria in a matrix of their own production. Many species of bacteria can adhere to surfaces and form biofilms. Although these biofilms are found in natural and industrial environments, they can cause resistant infections, particularly in hospitals. *Enterococcus* spp. are one of the common causes of opportunistic infections associated with biofilms. They are responsible for 25% of catheter-associated urinary tract infections and are often found in wounds. They are also increasingly being reported in cases of infectious endocarditis, all of which are associated with biofilms. Enterococcal biofilms are inherently tolerant to antimicrobial agents and therefore represent a serious obstacle to the treatment of infections. In addition to being difficult to eradicate, biofilm-associated enterococcal infections are a source of bacterial dissemination and a reservoir for antibiotic resistance genes (Ch'ng et al., 2019; Stoodley et al., 2002).

Within biofilms, microorganisms modify their behaviours when they detect that their population concentration has reached a certain level, then they begin to communicate through small signalling molecules. This mechanism is known as quorum sensing (QS) and is used to regulate genes associated with vir-

ulence, biofilm formation, antibiotic resistance, etc. Numerous natural and synthetic compounds can behave as anti-QS molecules by targeting QS systems to control biofilms (Shrestha et al., 2022).

As a result of the increase in antibiotic resistance, especially in recent years, the need for new antimicrobial agents is on the rise. Antimicrobial peptides (AMPs) play an important role in the innate immune system and have been investigated for their use in treating infectious diseases. However, their susceptibility to protease degradation, difficulty in large-scale production, and relatively high cost are among the difficulties encountered. Ceragenins (CSAs) are a group of antimicrobial agents that mimic the mechanisms of action of AMPs while eliminating these disadvantages. They are relatively easy to produce on a large scale and are not substrates for proteases as they are not peptide-based. They cause membrane depolarisation by forming transient pores in the membrane and cause cell death. In addition to their broad-spectrum antibacterial effects, they have strong antibiofilm activity. In previous studies, ceragenins were found to be effective against resistant Gram-positive bacteria, and these bacteria did not readily develop resistance to these molecules even after prolonged exposure. These findings suggest that ceragenins may be effective in the treatment of infections caused by drug-resistant *Enterococcus* spp. (Epand et al., 2007; Hacıoğlu et al., 2023). The aim of the present work is to investigate the *in vitro* antibiofilm, anti-QS, and antioxidant activities of ceragenins (CSA-44, CSA-90, and CSA-192) and linezolid against VRE isolates.

MATERIALS AND METHODS

Bacterial strains

A collection of 17 VRE isolated from various clinical specimens, including blood (n=9), urine (n=4), rectal swab (n=3), and vaginal fluid (n=1) was submitted to the Synevo Laboratories Ankara Central Laboratory in Türkiye. *E. faecalis* ATCC 29212 was used as a quality control strain.

Media and solutions

Before all experiments, each isolate was cultured on tryptic soy agar (Difco Laboratories, Detroit, MI, USA). Brain-heart infusion broth (BHIB, Thermo Scientific, Oxoid, USA) was used for the biofilm assays.

Antimicrobial agents

CSA-44, CSA-90, and CSA-192 were synthesized from a cholic acid scaffold method as described previously (Dao et al., 2020; Lai et al., 2008). Linezolid was kindly provided by its manufacturer, Koçak Farma İlaç ve Kimya Sanayi A.Ş. Stock solutions of ceragenins and linezolid were prepared from dry powders in distilled water. Frozen solutions of antimicrobial agents were used within six months.

Biofilm attachment assay

Biofilm attachment assay was performed using a previously described method with some modifications. Overnight cultures of isolates were prepared to a cell density equivalent to 1×10^6 cfu/mL as described above. Clinical isolates of VRE were added to each well of 96-well tissue culture microtiter plates containing 1/10x MIC of CSAs and linezolid, according to the MIC values determined in a previous study conducted by our working group, and the MIC results are presented in the supplementary material (Hacıoğlu et al., 2023). A positive control without antimicrobials and a negative control without cells were also prepared. Plates were incubated at 37°C for 1, 2, and 4 hours. After incubation, the wells were washed twice with physiologically buffered saline (PBS) and measured spectrophotometrically at OD 450 nm on a microplate reader (EON, Biotek, USA) (Hacıoğlu et al., 2021).

Inhibition of biofilm formation

Clinical isolates of VRE (1×10^6 cfu/ml) were added to each well of 96-well tissue culture microtiter plates containing 1x MIC, 1/10x MIC, and 1/100x MIC of CSAs and linezolid, positive control without antimicrobial, and negative control without cells. Plates were incubated at 37°C for 24 hours. At the end

of the incubation period, the wells were washed twice with PBS and measured spectrophotometrically at OD 450 nm using a microplate reader (EON, Biotek, USA) (Hacıoğlu et al., 2021).

Biofilm formation

Suspensions of $1x10^6$ cfu/ml were prepared from the overnight cultures of VRE isolates by dilution in BHIB. The prepared bacterial suspensions were transferred to 96-well microtiter plates and incubated at 37°C for 24 hours to achieve biofilm formation.

Effects of ceragenins and linezolid on mature biofilms

Biofilms were formed as described above to evaluate the activities of ceragenins at concentrations between 256-16 µg/mL and linezolid 64-4 µg/mL, on 24-hour-old mature VRE biofilms. Metabolic activities of the biofilms were assessed using the standardized static microtiter plate model and measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay. After biofilm formation, the medium was removed using a multichannel micropipette, and the wells were washed with PBS. CSAs were added to the wells, and the plates were incubated at 37 °C for 24 hours after positive and negative controls were prepared. After incubation, the wells were washed again with PBS. A solution of MTT at a concentration of 5 mg/mL was prepared. MTT solution was added to the wells and the plates were incubated for 3 hours at 35°C in the dark. Formazan crystals formed at the end of the incubation were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and measured spectrophotometrically at OD 450 nm using a microplate reader (EON, Biotek, USA) and evaluated by comparison with the control (Sabaeifard et al., 2014).

Anti-QS assay

The anti-QS activity of ceragenins (50, 25, 12.5 µg/mL) was examined using the biosensor strain *Chromobacterium violaceum* (*C. violaceum*) ATCC 12472. The anti-QS activity was investigated by means of a disk diffusion assay in Luria–Bertani agar (Sigma-Aldrich, St. Louis, MO, USA) plates with the appearance of a colorless ring (Hamidi et al., 2020).

Antioxidant capacities

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH scavenging activities of the ceragenins were determined using a modified Brand-Williams method (Brand-Williams et al., 1995), as described by Hasbal et al. (2015). Specifically, a 10 µL aliquot of each sample solution was combined with 240 µL of a 0.1 mM DPPH solution prepared in methanol. The resulting mixtures were subjected to agitation and subsequently incubated in darkness at ambient temperature for a duration of 30 min. Following incubation, the reduction in absorbance was quantified at a wavelength of 517 nm. Quercetin served as the reference standard. The percentage of DPPH radical scavenging activity was then calculated using the following formula:

$$DPPH\ scavenging\ activity\,(\%) = 1 - \left(\frac{Absorbance\ of\ the\ extract\ at\ 517nm}{Absorbance\ of\ the\ control\ at\ 517nm}\right) \times 100$$

Ferric-reducing antioxidant power (FRAP) assay

The antioxidant capacities of the ceragenins were quantitatively assessed using a modified FRAP assay, as described by Hasbal et al. (2015), based on the methodology outlined by Benzie and Strain (1996). The FRAP reagent was freshly synthesized by combining 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution (in 40 mM hydrochloric acid), and 20 mM ferric chloride solution in a volumetric ratio of 10:1:1. A 10 µL aliquot of each sample solution was combined with 20 µL of solvent, and the mixture was incubated at 37 °C for 10 min. Subsequently, 270 µL of FRAP reagent was added. Following a 4-minute incubation period, the increase in absorbance was measured at a wavelength of 593 nm. Quercetin was employed as the reference standard. The reducing capacity was expressed as FRAP values, represented as mM Fe^{2+} equivalents, derived from the

standard regression curve (y=0.5815x-0.0043, R^2=1) generated using ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) solutions within a concentration range of 0.1 to 1.5 mM.

RESULTS

Inhibition of adhesion

Among the ceragenins, CSA-44 and CSA-192 were the most effective, with adhesion inhibition rates of 18.18% and 20.91% after 1 h incubation, respectively, while CSA-90 was effective with an adhesion inhibition rate of 9.43% after 4 h incubation. It was found that linezolid was not as effective as ceragenins at 1 and 2 h. However, it was more effective than ceragenins after 4 h incubation with an inhibition rate of 11.18% (Figure 1.).

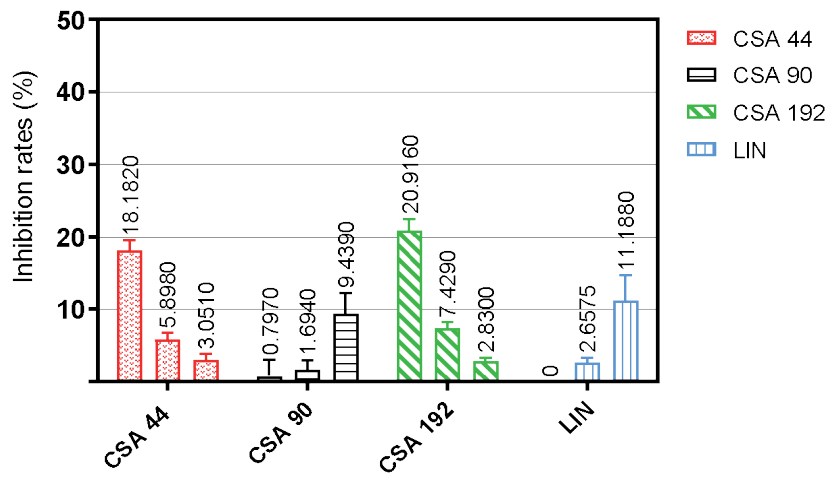

Figure 1. Inhibition of adhesion rates of antimicrobials after 1, 2, and 4 h incubation at MIC/10. The error bars indicate the standard deviations.

Inhibition of biofilm formation

Inhibition of biofilm formation rates depended on concentration; the highest inhibition rates were shown at MICs for all agents, as expected. CSA-44 and CSA-192 were the most efficient agents with inhibition rates of biofilm formation of 37.21 and 39.31 (%), respectively, at their respective MICs. Linezolid and CSA-90 showed similar inhibition rates at the MICs, while all three ceragenins were more effective than linezolid at the sub-MIC level. Results are shown in Figure 2.

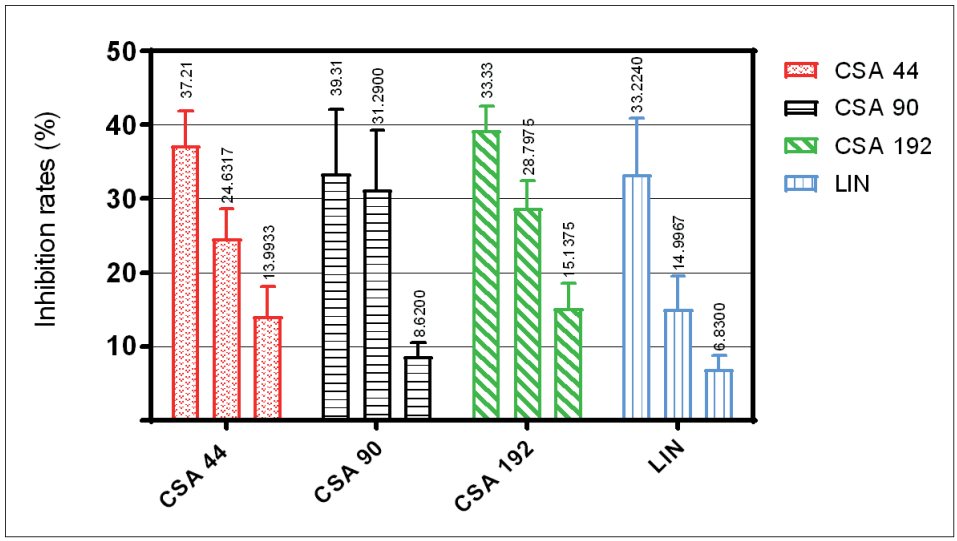

Figure 2. Inhibition of biofilm formation rates of antimicrobials at MIC, MIC/10, and MIC/100 concentrations, respectively. The error bars indicate the standard deviations.

Effects on mature biofilms

The results showed that CSA-44, CSA-90, and CSA-192 inhibited mature biofilms of VRE, at 16 µg/mL, by approximately 30, 39, and 20% respectively. In addition, at 32 µg/mL, CSA-44 and CSA-192 showed an inhibition of almost 50%, while CSA-90 showed an inhibition of almost 66%. In line with expectations, the inhibition rates increased as the concentration of ceragenins increased. Depending on the concentration, linezolid showed an inhibition rate between 39% and 60% against VRE (Figure 3.).

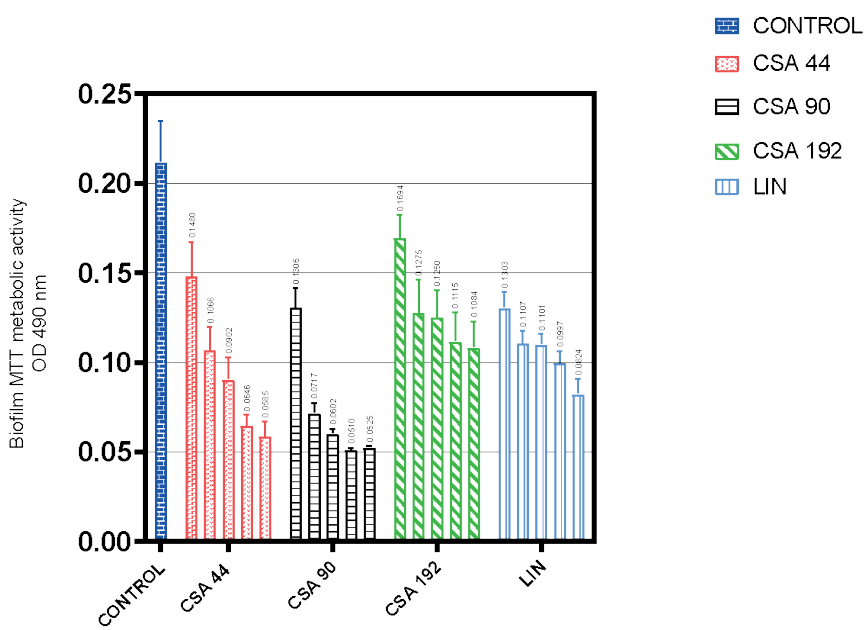

Figure 3. Effects of antimicrobials on mature biofilms, by the MTT method. Bars indicate 16, 32, 64, 128, and 256 µg/mL for ceragenins, 4, 8, 16, 32, and 64 µg/mL for linezolid. 256-16 and 32-2 µg/mL, on 24-hour-old mature VRE biofilms. The error bars indicate the standard deviations.

Anti QS effects of CSAs

In this study, the QS inhibition of *C. violaceum* 12472, a biosensor strain, by ceragenins was investigated using the disk diffusion method, and no QS inhibitory activity of ceragenins was detected at the concentrations studied.

Antioxidant effects of CSAs

The antioxidant capacities of the ceragenins were investigated using both DPPH radical scavenging activity and FRAP assays. For the DPPH radical scavenging activity assay, the samples were evaluated at concentrations of 100, 32, 16, 8, 4, 2, and 1 µg/mL. However, no detectable DPPH radical scavenging activity was observed across these concentrations. Similarly, under the tested concentrations, the ceragenins exhibited no ferric reducing antioxidant power, and FRAP values were undetectable. In contrast, quercetin, at a final concentration of 10 µg/mL, exhibited a DPPH radical scavenging activity of 87.437±0.175%. Furthermore, quercetin at a concentration of 125 µg/mL demonstrated a FRAP value of 2.312±0.143 mM Fe^{2+} equivalents.

DISCUSSION

Enterococci, members of the gastrointestinal microbiota, are known to be opportunistic pathogens, particularly in critically ill and immunocompromised patient groups. Since the emergence of VRE in the late 1980s, clinicians have faced significant challenges in the management of infections caused by these pathogens. Although they have relatively low virulence potential, their inherent tolerance and ability to establish persistent adaptation to common broad-spectrum antimicrobials have led to enterococci becoming a leading cause of healthcare-associated infections. In addition, resistance continues to emerge against new antibiotics such as daptomycin and linezolid, which have good treatment efficacy (Miller et al., 2020; Weiner-Lastinger et al., 2020). Moreover, the increase in biofilm-associated infections, which are known to be highly resistant to antimicrobial agents, and limited treatment options, reveal the need for new antimicrobial agents.

Biofilm, the form in which bacteria can live in complex communities embedded in the polymeric matrix they form by adhering to surfaces, is relatively common. Enterococci have an enhanced ability to adhere and therefore to form biofilms. These characteristics generally make biofilm removal difficult. Due to the increased binding surface, formation characteristics, and the polymicrobial nature of the biofilm itself, removal of mature biofilms is generally more difficult. In addition, previous studies have shown that biofilms play a role in most nosocomial enterococcal infections (Weiner-Lastinger et al., 2020). In line with emerging areas of research focusing on the prevention of adherence and biofilm formation of microorganisms, in the present study, we investigated the inhibition of biofilm attachment and biofilm formation by CSA-44, CSA-90, CSA-192, and linezolid against clinical VRE isolates. We found that CSA-44 and CSA-192 were more efficient in inhibiting adhesion after 1 h incubation, whereas CSA-90 and linezolid were more efficient after 4 h incubation. CSA-44 and CSA-192 were found to be the most efficient agents with inhibition rates of biofilm formation (after 24 h incubation) of 37.21 and 39.31 (%), respectively, at the MIC, and all three ceragenins were more effective than linezolid at sub-MIC levels. CSA-192 was the most effective in inhibiting adhesion and biofilm formation. In the study by Tekintaş et al. (2024), inhibition of biofilm formation of VRE isolates by CSA-44 was assessed at MIC (2-4 µg/ml) and MIC/2 concentrations. Higher inhibition rates were observed at the MIC concentration, with the inhibition of biofilm formation rates in the range of 49-97 (%) (Tekintaş et al., 2024). Another study observed the antibiofilm activity of CSA-44 against *E. faecalis* and *C. albicans* and found that CSA-44 inhibited biofilm formation and decreased the amount of biofilm formed by these microorganisms on teeth and dental composite (Tokajuk et al., 2022).

Since mature enterococcal biofilms are inherently resistant to antimicrobial agents, there is a great need

for new treatment options, as current antimicrobial agents are often inadequate for the treatment of biofilm-associated infections. Therefore, in our study, we also evaluated ceragenins and linezolid at different concentrations against mature VRE biofilms. CSA-44 and CSA-192 showed an inhibition of almost 50%, while CSA-90 showed an inhibition of almost 66% at a concentration of 32 µg/mL. As expected, the inhibition rates increased with increasing concentration of ceragenin. Depending on the concentration, linezolid showed an inhibition rate between 39% and 60%. Several studies have demonstrated the potent antibiofilm activity of CSA-44 in the concentration range of 10-20 µg/ml against mature biofilms formed by *E. faecalis* (Czarnowski et al., 2024; Tokajuk et al., 2022).

Although previous studies have also shown that CSA-44 inhibited the adhesion and biofilm formation of various microorganisms, such as *Achromobacter spp*. (Damar-Çelik et al., 2021), *Pseudomonas aeruginosa* (Bozkurt-Güzel et al., 2019), *Escherichia coli* (Bozkurt-Güzel et al., 2018), *Klebsiella pneumoniae* (Chmielewska et al., 2020) and *Candida spp.* (Bozkurt-Güzel et al., 2018). In this study, we have found that other ceragenins, especially CSA-192, also have significant antibiofilm activity against biofilms of VRE at early stages and CSA-90 against mature VRE biofilms.

In the present study, CSA-44 and CSA-192 exhibited stronger inhibition of early-stage biofilm adhesion, whereas CSA-90 was more effective against mature biofilms. Ceragenins are based on the bile acid cholic acid and mimic the amphiphilic morphology of AMPs. They affect biofilm formation by lysing cells, damaging cell membranes, and leaking cell contents, thereby destroying bacterial and fungal cells. CSA-44 contains an eight-carbon chain, but it also contains esters rather than ether bonds. CSA-192 is closely related to CSA-13, which previous studies have shown to rapidly penetrate biofilms and cause cell death without significantly altering the extracellular matrix. The ability of CSA-44 and CSA-192 to reduce biofilm adhesion and formation observed here demonstrates that these agents can maintain their activity within the composing biofilm extracellular matrix. In contrast, CSA-90 has two short (5-carbon) alkyl chains and does not display the activity observed with single alkyl chains. This can be by the fact that ceragenins with ester substituents (e.g., CSA-44) tend to act at the initial steps of biofilm formation, whereas CSA-90 can more readily permeate biofilm matrices (Bozkurt-Güzel et al. 2018; Tokajuk et al., 2022).

As QS inhibition plays an important role in biofilm disruption by interfering with microbial communication, we investigated the anti-QS efficacy of CSAs. According to the results, although they showed antibiofilm activity, they did not show anti-QS activity. This can be explained by the fact that antimicrobial compounds can exert antibiofilm activity through a variety of mechanisms, including direct bacterial killing, extracellular matrix degradation, and inhibition of adhesion or metabolic pathways (Roy et al., 2018).

The antioxidant capacities of the ceragenins were also investigated using DPPH radical scavenging activity and FRAP assays. Oxidative stress describes the imbalance between the antioxidant defence mechanisms and the levels of reactive oxygen and nitrogen species. Antioxidants are compounds that are able to inhibit oxidation by neutralising the damaging effects of free radicals, and they protect key cellular components. While it is generally posited that elevated antioxidant levels correlate with enhanced antimicrobial activity (Ispiryan et al., 2024), the absence of antioxidant activity observed in ceragenins at the studied concentrations suggests a mechanism of action distinct from traditional antioxidant pathways. This finding prompts the hypothesis that ceragenins primarily exert their antimicrobial effects through membrane depolarization, and potentially may in-

volve contributions from oxidative stress, rather than solely relying on direct antioxidant activity. Given the complexity of antimicrobial compound mechanisms, where multiple pathways may operate concurrently, and the cationic nature of ceragenins, which might induce oxidative stress, further investigation into the possible interaction of ceragenins with oxidative stress pathways is warranted to fully elucidate their antimicrobial mechanisms.

CONCLUSION

The emergence and spread of VRE presents a growing issue in clinical microbiology and infectious disease management and requires new antimicrobial agents. According to the present study, ceragenins appear to be potential agents with antibiofilm activity against VRE strains. Due to its high resistance to antimicrobial agents, biofilm treatment is extremely challenging. Promisingly, in biofilm assays, ceragenins were found to be more effective than linezolid, which is commonly used to treat infections but to which resistance is increasing. However, more research is needed to fully determine their effectiveness and safety in clinical use. This study highlights the need for continued research in this area and suggests that ceragenins may be a potential treatment option the combat against VRE.

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AUTHOR CONTRIBUTION STATEMENT

Concept – MA, FO, MH; Design – MA, FO, MH; Supervision – MA, FO, MH; Resources – MH; Materials – MH, N., PBS; Data Collection and/or Processing – MA, FO, MH, GHC, NI; Analysis and/or Interpretation – MA, FO, MH, GHC, NI, PBS; Literature Search – MA, FO, MH; Writing – MA, FO, MH, GHC, NI, PBS; Critical Reviews – MA, FO, MH, GHC, NI, PBS.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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