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ORIGINAL ARTICLE

Effect of Chlorhexidine (CHX) and Hydrogen Peroxide (H_2O_2) on the Biofilm Formation of *Enterococcus faecalis*

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SUMMARY

Background: **Biofilm makes bacteria resistant to antimicrobial agents and facilitates the transmission of infectious diseases in hospitals. Disinfectant compounds are frequently used to control surface contamination. This study was designed to investigate the effect of chlorhexidine (CHX) and hydrogen peroxide (H_2O_2) on biofilm formation of *Enterococcus faecalis*.**

Methods: **This study was performed on 40 *E. faecalis* clinical isolates. After the determination of MIC, the effect of different concentrations of CHX and H_2O_2 on the biofilm formation was evaluated. Also, the relative expression level of the studied biofilm genes, following exposure to sublethal concentration of CHX and H_2O_2, was assessed using quantitative reverse transcription PCR (qRT-PCR).**

Results: **The frequency of the *asa1, efaA, epaI, and esp* biofilm genes were 80%, 92.5%, 100%, and 75%, respectively. Various concentrations of CHX increased the biofilm mass in *E. faecalis*. Also, the combination of CHX and H_2O_2 at sub-minimal inhibitory concentrations, significantly elevated the expression of *asa1, epaI,* and *esp* genes.**

Conclusions: **The results of this study showed that the improper use of disinfectants can increase the ability of biofilm formation in *E. faecalis* and may cause selective pressure leading to the emergence of biocide-resistant microorganisms.**

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INTRODUCTION

Biofilm is a complex structure of microbial cells in which the cells attach to the surrounding environment. These cells are embedded in extracellular polymeric substances, a milieu that is mostly composed of polysaccharides, extracellular DNA (eDNA), and proteins, which renders bacteria resistant to antibiotics [1].

Biofilms are recalcitrant to antibiotic therapy and are a major cause of persistent and recurrent infections, especially in nosocomial settings through indwelling devices.

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The National Institutes of Health (NIH) declared that, respectively, 65% and 80% of all microbial and chronic infections are associated with biofilm formation [1]. For instance, in the United States in 2002, a total of 1.7 million people were infected with nosocomial infections and approximately 99,000 deaths were reported [2]. Most accessible antimicrobial treatments target the microorganisms in the planktonic (free-living) mode of life [3]. Thus, these treatments are often ineffective against pathogenic biofilms [3,4]. Today, different chemical and physical methods are used to inactivate or destroy microorganisms in medical settings, among which disinfectants are most common [3,4]. While these biocide treatments eradicate most surface contaminations, some microorganisms may endure even high concentrations of these compounds, proliferate and give rise to substantial problems in terms of public health [3]. It seems that the resistance of microorganisms to disinfectants is frequently related to biofilm formation which provides the microorganisms with the condition and time needed to acquire resistance genes and elements from the environment or to mutate genes accordingly [5]. The improper use of disinfectants may accelerate these processes of resistance. In fact, sublethal concentrations of these compounds can act as a signal to stimulate biofilm formation [6].

Enterococci have arisen as a significant nosocomial pathogen and are second only to staphylococci as a cause of Gram positive nosocomial infection worldwide [7]. Enterococci are also the second most frequent cause of nosocomial urinary tract infection, infective endocarditis, and bacteremia. Moreover, they are also associated with intra-abdominal and pelvic infections, gastrointestinal infections, and oral infections [8,9]. A significant increase in the prevalence of vancomycin-resistant enterococci (VRE) has been reported recently in many countries [7]. The capability of enterococci to withstand hostile conditions, the presence of a repertoire of virulence factors, as well as inherent and acquired antibiotic resistance traits poses therapeutic challenges for clinicians to treat infections caused by this bacterium [8,9]. Therefore, the main aim of the present study was to survey the effect of chlorhexidine (CHX) and hydrogen peroxide (H_2O_2), as common disinfectant agents, on the expression level of biofilm-related genes in *Enterococcus faecalis.*

MATERIALS AND METHODS

Bacterial collection and identification

In this cross-sectional study, a total of 180 clinical samples were collected from Tehran and Ahvaz hospitals, Iran.

The samples were immediately transferred to the Research Center of Ilam University of Medical Sciences, Iran.

Culture and biochemical tests

For isolation and phenotypic identification of *Enterococcus* species, samples were cultured on blood agar medium and incubated at 37°C for 24 hours. Enterococci were identified to the genus level using Gram staining, catalase and bile esculin tests, as well as culture in brain heart infusion medium containing 6.5% sodium chloride. To identify enterococci to the species level, the arabinose fermentation test was performed. In this context, the yellow color of the broth medium indicated a positive reaction. Isolates with a negative reaction were considered as *E. faecalis*.

DNA extraction and PCR amplification

The total DNA was extracted using the Genomic DNA Extraction Kit (Bioneer, The Republic of Korea). The presence of *Asa1*, *epaI*, *efaA*, and *esp* genes in the studtied isolates were tested using PCR method. Primers are shown in Table 1. The PCR reaction mixture was prepared in a final volume of 20 µL using the following procedure: initial denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing temperature (Tm of each primer is shown in Table 1) for 30 seconds, and extension at 72°C for 20 seconds. A final extension step was performed at 72°C for 4 minutes.

Determination of minimum inhibitory concentration (MIC)

To determine the MICs of chlorhexidine (Morvaben Co.) and H_2O_2, 100 mL of 0.5 McFarland overnight bacterial culture (dilution of 1:100 in Mueller Hinton broth) and different concentrations of antibiotic solution were added to the 96-well microtiter plates and incubated at 37°C for 18 hours. The MIC was considered as the lowest concentration of antibiotic that inhibited visible bacterial growth after 18 hours of incubation. MIC tests were repeated three times [10].

Biofilm formation assay

To evaluate the ability of biofilm formation, exponential and stationary phase cultures of *E. faecalis* were diluted to an optical density at 600 nm (OD_{600}) of 0.08 in 1% glucose trypticase soy broth (TSB) medium (Merck, Darmstadt, Germany) and 200 µL of the bacterial suspension were inoculated in each well of 96-well plates. The plates were then incubated at 37°C for 24 hours. In the next step, the wells were washed twice with PBS and stained by 100 µL of 1% crystal violet for 20 minutes. This process was followed by washing twice with PBS and transferring 100 µL of 95% ethanol to each well. The plates were then incubated at room temperature for 15 minutes. The solubilized crystal violet and the cell density were quantified by measuring OD at 570 nm by the microplate reader Varioskan Flash (Thermo Scientific, Billerica, MA, USA). The Biofilm assay was repeated three times for both isolates. The 1% glucose TSB medium without bacteria served as the negative control. The optical density cutoff (ODc) was

determined as three standard deviations higher than the mean OD of the negative control. All the isolates were grouped into four groups based on the adherence capabilities: non-biofilm-former (OD ≤ ODc), weak biofilm former (ODc < OD ≤ 2 x ODc), moderate biofilm former (2 ODc < OD ≤ 4 x ODc), and strong biofilm former (OD > 4 x ODc). Moreover, to investigate the effect of CHX and H_2O_2 on the biofilm formation, 100 µL of different concentrations of these compounds were added to 96 well-microtiter plates and the aforementioned steps were performed. The control sample was placed in a microtube containing TSB (without glucose) at 37°C [11].

Quantitative Real-Time PCR (qRT-PCR)

To investigate the effect of CHX and H_2O_2 on the expression level of the *asa1*, *efaA*, *epaI*, and *esp* genes, the real-time PCR method was performed. Briefly, isolates with the ability of strong biofilm formation were exposed to sub-MIC concentrations of the investigated biocides at 37°C, and after 1 hour, the RNA extraction was performed using the trizol solution. RNA samples which had an OD 260/280 ratio between 2 and 2.15 were used for cDNA synthesis using Takara cDNA Synthesis kit. Finally, the expression level of the genes was assessed by real-time PCR. The *16srRNA* gene was used as the internal control. The relative fold changes in expression levels were calculated using the Livak formula [12].

Statistical data analysis

Significant differences were assessed using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test and Dunnett's test for multiple comparisons (as indicated in the figure legends). All data analysis and statistical chart drawings were carried out using GraphPad Prism 8 (GraphPad Software, Inc).

RESULTS

Identification and confirmation of *E. faecalis*

A total of 53 *E. faecalis* isolates were identified using phenotypic methods while only 40 isolates were confirmed to be *E. faecalis* using the PCR technique (Figure 1).

Frequency determination of the *efaA, asa1, epaI,* and *esp* genes using the PCR method

The prevalence of the *asa1*, *efaA*, *epaI*, and *esp* genes in 40 *E. faecalis* isolates was found to be 80%, 92.5%, 100%, and 75%, respectively (Figure 2).

Biofilm formation in the presence of MIC and sub-MIC concentrations of 0.01% chlorhexidine

The MIC of 0.01% chlorhexidine gluconate and 2% H_2O_2 and their combination on *E. faecalis* was 1:32 (0.000003 g/mL), 1:64 (0.0003 g/mL), and 1.128 g/mL in initial concentration, respectively.

The results of the biofilm assay demonstrated that *E. faecalis* produced thicker biofilms, even thicker than the positive control samples, at sub-MIC concentrations of chlorhexidine. Biofilm growth was stopped at a concentration of 0.000003 g/mL of this biocide (Figure 3).

Inhibition of biofilm formation by different concentrations of hydrogen peroxide

Different concentrations of hydrogen peroxide were added to wells containing biofilm and incubated at 37°C for 24 hours. Finally, their OD was measured at 630 nm with an ELISA reader. The results of biofilm assay demonstrated that all the concentrations of hydrogen peroxide almost uniformly inhibited the biofilm formation of *E. faecalis* and its planktonic growth. Higher concentrations of hydrogen peroxide did not show stronger anti-biofilm properties (Figure 4).

Relative qRT-PCR

The results of the qRT-PCR assay revealed that the expression level of the *asa1*, *epaI*, and *esp* genes in *E. faecalis* was significantly increased in the presence of sub-lethal doses of CHX and H_2O_2 and their combination (Figure 5). Although all the studied concentrations showed, to some extent, a stimulatory effect on the expression of the studied biofilm genes, the combination of CHX and H_2O_2 showed the highest stimulatory effect on these gene expressions. Expression levels of *esp*, *asa1*, and *epaI* genes showed different changes in the presence of disinfectants. A significant increase was not observed in the expression level of the *efaA* gene.

DISCUSSION

Nosocomial infections are associated with high mortality and hospitalization rates and have large financial impacts on hospital resources [13]. These infections extend the length of hospital stay and result in decreased total throughput of patients [14].

Biofilm formation is a survival strategy for bacteria which helps them become compatible with their environment such as medical setting [15]. Today, researchers believe that the biofilm structure is the main route of nosocomial infection in which the microorganisms can become persistent [15]. Enterococci are the second leading cause of nosocomial urinary tract infection, bacteremia, and infective endocarditis [16]. Although different methods are used to control microbial contamination, the prevalence of nosocomial infections is increasing and has become a major concern in medical health system [17]. CHX and H_2O_2 are the most commonly used antimicrobial agents in hospitals and medical centers [17]. In the present study, the effects of sub-lethal concentrations of CHX and H_2O_2 on the ability of biofilm formation and the expression level of biofilm-related genes in *E. faecalis* were investigated.

The sub-minimum inhibitory concentrations (sub-MICs), i.e., the concentrations lower than the MIC val-

Table 1. Characterization of the primers used in this study.

Genes	Primer sequence (5′→3′)	Tm (°C)	PCR product Size (bp)
16SrRNA	F: CCGAGTGCTTGCACTCAATTGG R: CTCTTATGCCATGCGGCATAAAC	60	137
Asa1	F: GATACAAAGCCAATGTCGTTCCT R: TAAAGAGTCGCCACGTTTCACA	58	101
epaI	F: GCGGACGATTGCTTCCATTG R: TTGGTTCCATCTGTCGAGCC	60	102
efaA	F: TGGGACAGACCCTCACGAATA R: CGCCTGTTTCTAAGTTCAAGCC	60	101
esp	F: GCATCAGTATTAGTTGGT R: TTCCTTGTAACACATCAC	60	196

PCR - polymerase chain reaction, F - forward, R - reverse.

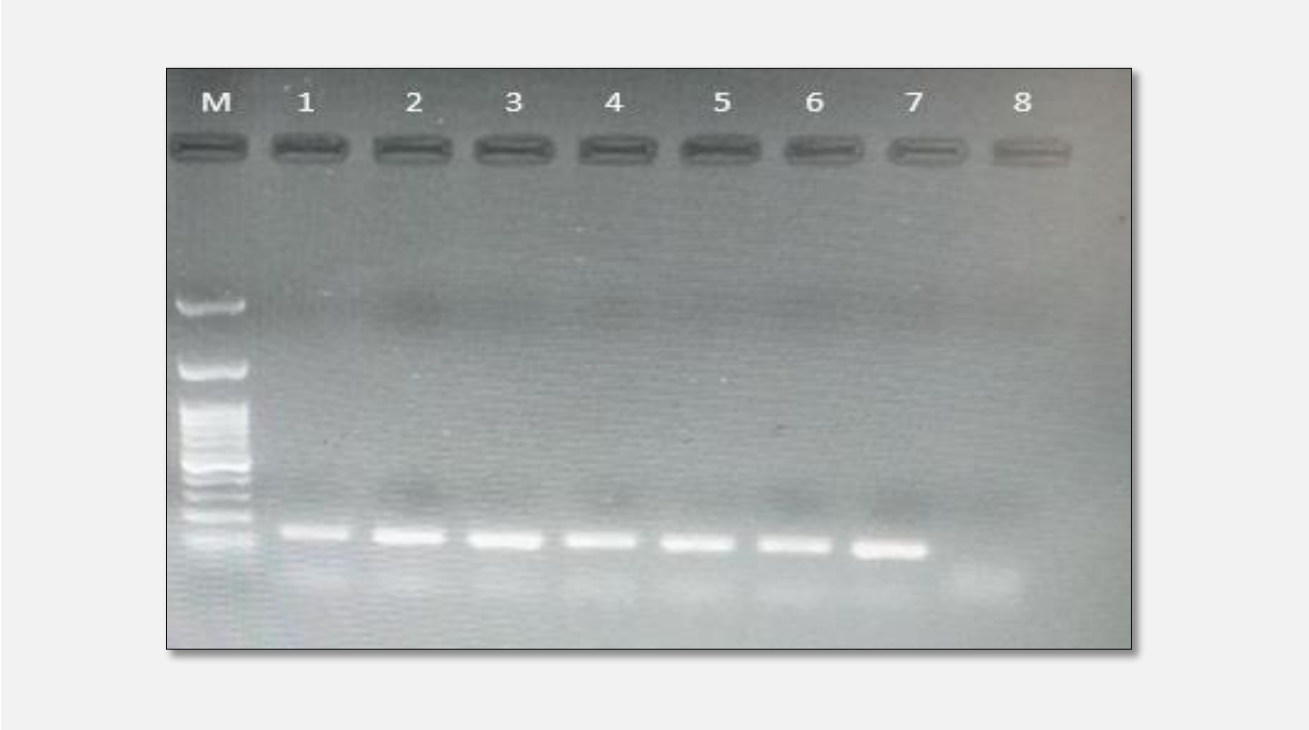

Figure 1. PCR result of *E. faecalis* identification using the *16srRNA* gene, M - size marker = 100 bp, Lane 7 - positive control, Lane 8 - negative control.

ues, have been declared, in case of antibiotics, to act as signaling molecules which, although not able to inhibit the bacterial growth, can still alter the physicochemical characteristics and expression level of the bacterial virulence factors and reduce the overall bacterial virulence [18]. The changes caused by the sub-MIC concentrations of antibiotics are indicative of a condition that bacteria encounter in a wild environment and the way that they deal with it [18]. The results of PCR technique showed that phenotypic methods may not be very accurate and it is better to use molecular methods to confirm the strains.

The results of this study showed that most of the studied enterococcal strains have the ability to form biofilms and that the sublethal concentrations of CHX and H_2O_2 can induce and intensify the formation of biofilms. Moreover, analysis of the relative gene expressions in the presence of sub-MIC concentrations of CHX and

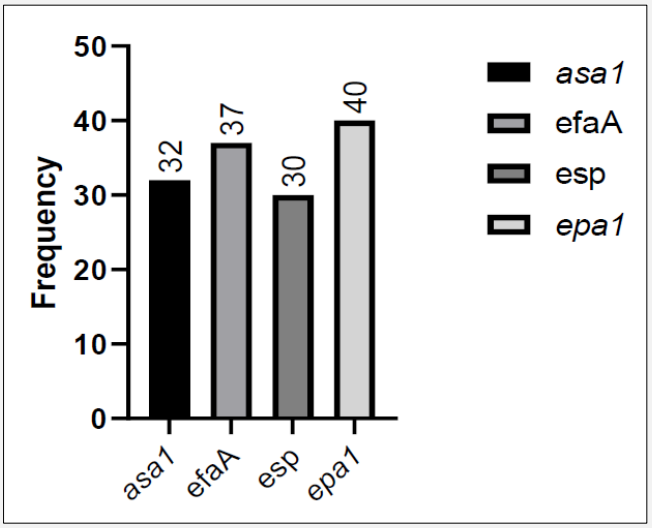

Figure 2. Frequency of the *asa1, efaA esp,* and *epaI* genes among 40 *E. faecalis* isolates.

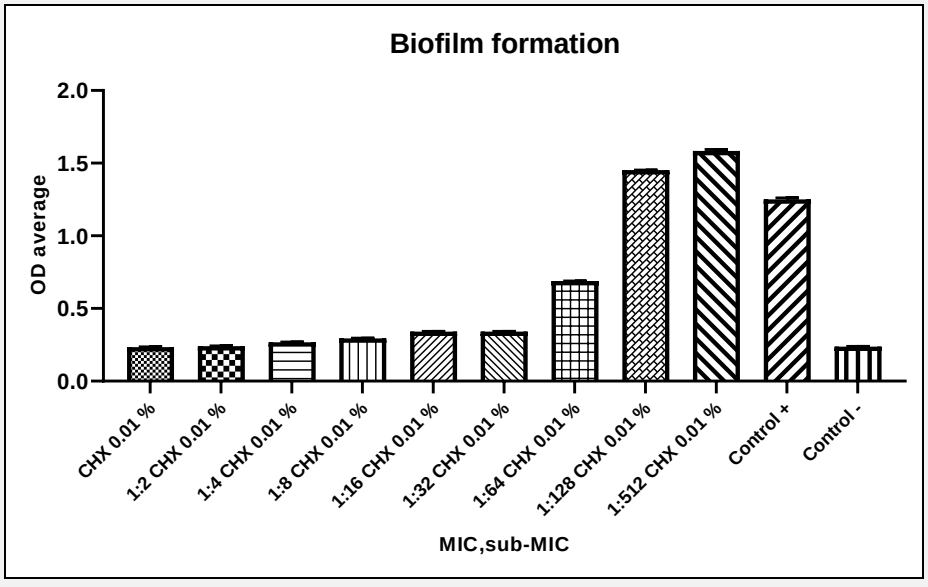

Figure 3. Biofilm formation in the presence of MIC and Sub-MIC concentrations of chlorhexidine.

H_2O_2 and their combination, demonstrated up-regulation of the *asa1*, *epaI*, and *esp* biofilm genes, indicating their role in biofilm formation. The sub-MIC concentrations of the disinfectants used in this study may act as a signal for biofilm formation in *E. faecalis* through simulation of the two-component signal transduction systems (TCSs) [19].

Pauline et al. in 2017 demonstrated the prevalence of

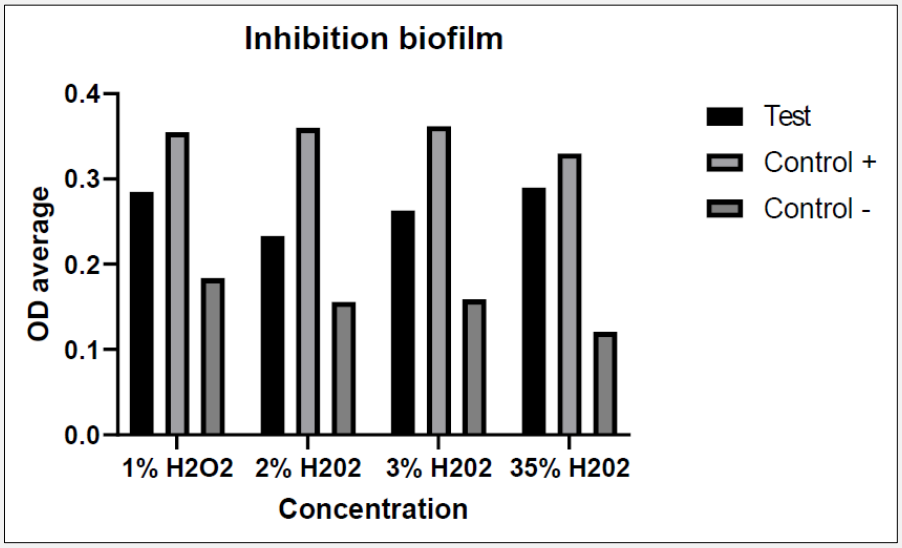

Figure 4. Inhibition of biofilm formation in the presence of different concentrations of hydrogen peroxide.

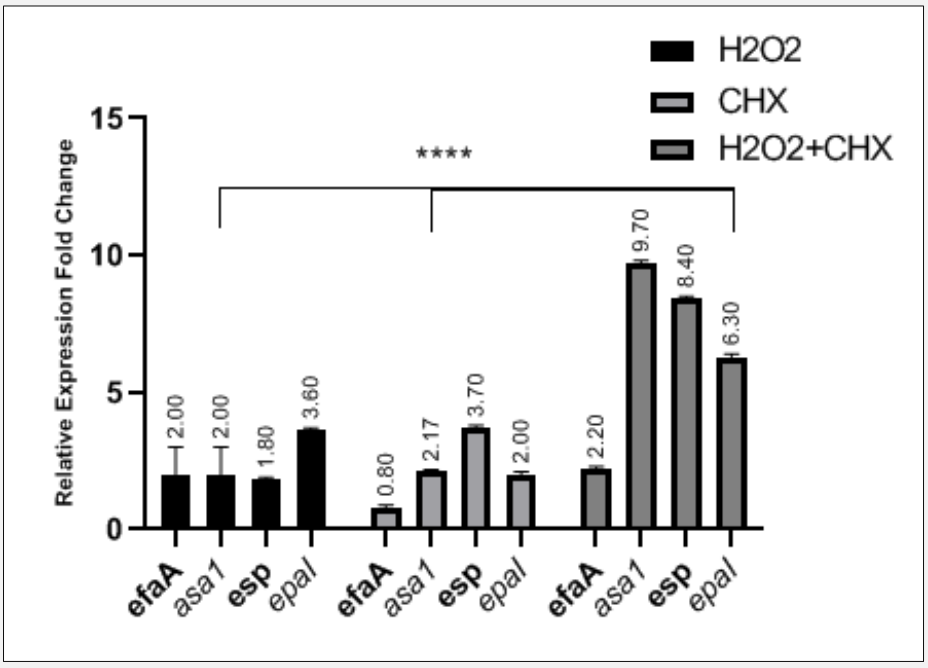

Figure 5. Comparison of the effect of chlorhexidine gluconate and hydrogen peroxide on the expression level of the *efaA*, *asa1*, *epaI*, *and esp* genes in *E. faecalis*. Data represents the mean (± SD) of three biological replicates. One-way ANOVA with Tukey's post-hoc test was used for multiple comparisons.

* - p < 0.05, ** - p < 0.01, *** - p < 0.001.

antiseptic-resistance quaternary ammonium compound (QACs) genes in staphylococci [20]. Their result showed that *qacA*/*B* was the predominant gene detected, being present in 26.5% of coagulase negative staphylococci (CNS) and 11% of *S. aureus* isolates. The *smr* and *qacH* genes were present in 12% of the CNS but were less common among *S. aureus* isolates. They stated that the long-term use of multipurpose solutions containing quaternary ammonium compounds may select for carriage of organisms harboring QAC genes [20]. In another study, it was shown that the frequency of antiseptic resistance genes was high (49/69; 71.0%) in clinical staphylococci isolates but absent (0/69; 0%) in enterococci isolates and that the MICs of benzalkonium chloride (BAC) and chlorhexidine digluconate (CHDG) against vancomycin-resistant enterococci (VRE) isolates were significantly higher than those against vancomycin-susceptible enterococci (VSE) isolates [21]. On the other hand, biofilm is one of the major causes of persistent infections, especially in nosocomial settings through indwelling devices. Similar to the present study, there is sufficient evidence that sublethal concentrations of antimicrobials can enhance biofilm formation in bacteria. For instance, in 2019, the effect of low doses of disinfectants on the biofilm-forming ability of *Listeria monocytogenes* was considered by Rudriguez-melcon et al. [22]. They showed that the concentrations close to the MIC of sodium hypochlorite (SHY) or benzalkonium chloride (BZK) can enhance the biofilm-forming ability of BZK-resistant *L. monocytogenes* strains. They emphasized the importance of avoiding sub-MIC disinfectant concentrations in food-handling environments [22]. A recent study has used similar compounds used in this study, to remove *E. faecalis* as a causative agent in endodontic diseases. In this study, researchers have indicated that 2.5% NaOCl and 2% chlorhexidine show considerable efficacy against *E. faecalis* while 1.5% hydrogen peroxide is not able to eradicate all the *E. faecalis* colonies [23].

In addition, Parolia et al. in 2021 investigated the effects of propolis nanoparticles (PNs) against *E. faecalis* biofilm in the root canal. In this study, two-hundred-ten extracted human teeth were sectioned to obtain 6 millimeters of the middle third of the root. The root canals were enlarged to an internal diameter of 0.9 mm. The specimens were inoculated with *E. faecalis* for 21 days. PN300 was as equally effective as 6% NaOCl and 2% CHX in reducing the *E. faecalis* biofilms [24].

CONCLUSION

The sublethal concentrations of disinfectants can increase the ability of biofilm formation in *E. faecalis* and can cause selective pressure leading to the emerging of biocide-resistant microorganisms. Therefore, the use of appropriate concentrations of antimicrobials should be carefully considered, and it is recommended to periodically and regularly examine microorganisms in terms of their resistance to common antimicrobials used in relevant settings.

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Declaration of Interest:
Authors declared no conflict of interests.

Ethical Approval:
The Ethics Committee of Ilam University of Medical Sciences approved the study protocol (code: 971024/61).

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