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Original Article

Unraveling the molecular regulation of biofilm underlying effect of chronic disease medications

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Abstract

A biofilm is a complex microbial structure that promotes the progression of persistent infections, particularly in nosocomial settings via indwelling medical devices. Conventional antibiotics are often ineffective treatments for biofilms; hence, it is crucial to investigate or design non-antibiotic antibiofilm compounds that can successfully reduce and eradicate biofilm-related infections. This study was an attempt to repurpose chronic disease medications of the antihypertensive and antilipidemic drug classes, including candesartan cilexetil (CC) and ursodeoxycholic acid (UDCA), respectively, to be used as antibiofilm agents against the two infectious pathogens Staphylococcus aureus and Enterococcus faecalis. Crystal violet (CV) staining assay was used to evaluate the antibiofilm activity of the drugs. Real-time polymerase chain reaction (RT-PCR) was performed to determine the transcription levels of the biofilm-related genes (icaA and icaR in S. aureus and fsrC and gelE in E. faecalis) following treatment with different concentrations of CC and UDCA. we found that a concentration of greater than 1.5 µg/ml of CC significantly ($p < 0.005$) inhibited the biofilm formation of both bacterial isolates, and a concentration of greater than 50 µg/ml of UDCA significantly ($p < 0.005$) inhibited the biofilm formation of both bacterial isolates. Interestingly, the mRNA expression levels of biofilm-related genes were decreased in the two bacterial isolates at concentrations that were lower than the human pharmaceutical daily doses.

Keywords: Biofilm gene, Regulation, *S. aureus, E. faecalis.*

1. Introduction

Bacterial biofilms are the main relevant driver of long-term infections and are a crucial concern in healthcare settings due to their resistance to antimicrobial agents, immune response, and environmental factors [1]. Clinically, biofilms remain a challenge, as they occur on different surfaces of organs, such as the exterior mucosal layer of the digestive and respiratory pathways and the skin, and on medical instruments, including various types of catheters, heart valves, pacemakers, breast implants, contact lenses, ventilation tubes, and prostheses [2]. In many instances, biofilm formation is the main cause of implant failure, particularly in immunocompromised and older patients, and surgical implant replacement is an effective method for eliminating infections [3, 4].

Here, two highly resistant strains of gram-positive bacteria, which are commonly used as biofilm model organisms, were selected: *Staphylococcus aureus* and *Enterococcus faecalis*. *S. aureus* is expected to cause multiple types of infections and biofilms on implanted medical devices, such as skeletal prostheses, prosthetic heart valves, and catheters [5]. *E. faecalis* has gained increased attention on a global scale as an alarming opportunistic pathogen because of its capacity to create biofilms and because it is a major cause of nosocomial infections [6]. Biofilm formation is a multistep process that requires the coexpression of several genes. The expression of these genes in each step, from initial adherence to dispersion, is tightly controlled by numerous regulators or regulatory systems [7]. The main exopolysaccharide produced by *Staphylococcus* biofilm is polysaccharide intercellular adhesin (PIA) or poly-N-acetyl glucosamine (PNAG). The *icaADBC* operon encodes the enzyme essential for PIA production. The regulation of biofilm formation relies strongly on the roles of the *icaA* and *icaD* genes. The *icaA* gene product is a transmembrane protein, an N-acetylamino-glucosamine transferase, and the *icaD* gene product is the chaperone protein of *icaA* [8]. Transcription of *icaADBC* has been shown to be regulated by various transcriptional regulators, including IcaR, TcaR, SarA, and the σ^B factor [9].

The *icaR* gene encodes a DNA-binding protein, a transcriptional repressor that adversely regulates the expression of i*caADBC* by binding the start codon of *icaA*'s upstream region. The *TcaR* (teicoplanin-associated locus regulator) gene also encodes a transcriptional repressor for the *ica* locus [10]. In addition to IcaR and TcaR, the two global

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response regulators SarA and σ^B regulate *S. aureus* biofilm formation. The SarA regulator is required for *ica* operon transcription, and the *sigB* operon encodes σ^B, which is the primary regulator of *S. aureus* in response to external stress and plays a significant role in antibacterial resistance [11].

A few *S. aureus* strains are capable of *ica*-independent biofilm formation using proteins rather than polysaccharides. This process is mediated by numerous surface adhesins, including the fibronectin-binding proteins FnBPA and FnBPB and the biofilm-associated protein Bap [12]. Bacteria in a biofilm employ an internal communication mechanism called a quorum-sensing system for initiation to respond to environmental changes, the accessory gene regulator (*agr*) locus and a communication substance called autoinducing peptide (AIP) constitute the *S. aureus* quorum-sensing system. The *agr* quorum-sensing system in *S. aureus* is involved in biofilm dispersal and colonization of new sites [8].

Several genes are described as being related to biofilm formation in *E. faecalis*. The most important genes are gelatinase (*gelE*) [13], *Asa* (aggregation substance), *Esp* (extracellular surface protein), *EfaA* (*E. faecalis* antigen A), *Ace* (adhesin of collagen from *E. faecalis*), *Ebp* (endocarditis and biofilm-associated pilli), and cytolysins (*cyl*) [14]. The gene *gelE* on the *E. faecalis* chromosome codes for a gelatinase, zinc-containing metalloproteinase. The *sprE* gene encodes a serine protease; it is located directly downstream of *gelE* and is co-transcribed with it. As a component of the *gelE–sprE* operon, the *gelE* gene, along with the *fsrABC* loci, is regulated positively by the *fsr* quorum-sensing system. Peptide accumulation in the extracellular environment is sensed by the FsrC histidine kinase, causing the activation of the response regulator FsrA [15]. The ability of gelatinase to break down the collagen protein, which aids in the bacteria's adhesion to both abiotic and biotic surfaces, is one of the enzyme's properties most important to biofilm development. Along with serine protease, gelatinase plays a critical role in N-acetylglucosaminidase (*AtlA*) regulation. This enzyme plays an essential role in the formation of the extracellular DNA that exists in a biofilm [16].

To our knowledge, only a few published studies have evaluated the effects of a number of antibiotics and environmental conditions on biofilm formation [17- 20]. Furthermore, their results remain controversial, as these approaches often fall short, leaving patients, particularly those who are older and have chronic diseases, with persistent harmful biofilms. Therefore, a potential solution for this alarming situation, and for combatting development of resistance in serious pathogens, would be identifying the antibiofilm activity among FDA-approved non-antibiotic chronic disease medications. Recently, several studies have reported bactericidal effects of these non-antibiotic drugs [21], but their effects on biofilms and the regulation of biofilm genes have not yet been studied. Thus, this study attempted to repurpose certain chronic disease medications of the antihypertensive and antilipidemic drug classes, including candesartan cilexetil (CC) and ursodeoxycholic acid (UDCA), respectively, as antibiofilm agents against the most infectious pathogens of the burdensome antibiotic-resistant biofilm-forming bacteria in hospital and community settings. The selection pressure for resistance may be lessened by this alternative strategy of suppressing biofilm without inhibiting bacterial growth.

The current study aimed to assess the *in vitro* antibiofilm activity of non-antibiotic drugs, CC and UDCA, against two gram-positive bacterial isolates: *S. aureus* and *E. faecalis.*

2. Materials and methods

2.1. Bacterial isolates

In this study, biofilm-positive strains of *S. aureus* and *E. faecalis* were borrowed from a previous clinical study (Majid *et al*., unpublished) that screened patients with indwelling medical devices for biofilm-positive bacteria.

2.2. Chemicals and materials

The studied drugs CC and UDCA were obtained from a Pioneer Company for Pharmaceutical Industries in Sulaimaniyah, Iraq, as pure powder. All chemicals and media were obtained from the basic medical science department of the College of Medicine, University of Sulaimaniyah, Iraq. An RNA extraction kit and RT-PCR components were supplied by New England Biolabs Company, Germany. The primers (HPLC purified) were supplied by Sigma Aldrich Company, Germany. The manufacturer's recommendations were followed for optimal handling and storage of these materials.

2.3. Evaluation of the effects of CC and UDCA on biofilm formation

To evaluate the antibiofilm activity of CC and UDCA, a crystal violet (CV) staining assay was conducted using a microtiter plate, as described by Stepanovic *et al*. (2007) [22] and Kafil *et al*. (2016) [23], with a few adjustments. Briefly, *S. aureus* and *E. faecalis* cells were transferred from the stock culture onto blood agar and incubated aerobically at 37 ℃ for 24 h. Then, two to four well-isolated colonies were suspended in 5 ml of sterile trypticase soya broth (TSB) containing 1% glucose and incubated at 37 ℃ for 18 h. Then, the bacterial suspension's turbidity was adjusted to match the 0.5 McFarland standard (1.5×10^8 CFU/ml). The bacterial inoculum obtained was diluted 1:100 in TSB supplemented with glucose.

To evaluate the antibiofilm activity of CC and UDCA, each well of a sterile 96-well polystyrene flat-bottom plate was filled with 180 µl of different concentrations of both CC and UDCA. Then, 20 µl of diluted (1:100) bacterial inoculum was added to the wells to obtain 0, 1.5, 3, 3.5, 4, 4.5, and 5 µg/ml of CC and 0, 50, 100, 150, 200, 250, and 300 µg/ml of UDCA. The TSB was poured into five wells, which were considered the negative controls. The covered plates were incubated at 37 ℃ for 24 h in a static condition. After incubation, the optical density (OD) of bacterial growth with the drug was measured at 630 nm (OD_{630}) using a microtiter plate reader. After discarding the supernatant, the wells were washed three times with sterile phosphate-buffered saline (PBS) to remove the detached cells. Then, the plates were fixed at 60 ℃ for 1 h. After fixation, the biofilms were stained with 200 µl of CV solution (2%) for 30 min, and the wells were washed with PBS. The microtiter plates were air-dried by inverting them on a paper towel. To resolubilize the CV, 150 µl of ethanol (98%) was added to each well, and the lidded plates were left at room temperature for 30 min without shaking. The OD of resolubilized CV was measured at 630 nm using a microtiter plate reader. This experiment

was performed in five wells for each concentration and repeated at least three times. The final OD value of each concentration for each bacterial strain was expressed as the obtained mean OD value following subtraction of the ODc value[20, 24].

ODc: Average OD of negative control + (3 × standard deviation (SD) of negative control)

OD isolate: Average OD of isolate − ODc

2.4. Assay of biofilm gene expression

Real-time polymerase chain reaction (RT-PCR) was performed (in a Real-time PCR system CFX96 Deep Well; BIO-RAD, U.S.A.) to determine the transcription levels of the biofilm-related genes (*icaA* and *icaR* in *S. aureus* and *fsrC* and *gelE* in *E. faecalis*) following treatment with different concentrations of CC and UDCA. The *16S rRNA* and *recA* genes for *S. aureus* and *E. faecalis*, respectively, were used as internal control genes or housekeeping genes to normalize the PCRs. Briefly, 20 µl of diluted (1:100) bacterial inoculum was added into each well of a 96-well microtiter plate containing 180µl of different concentrations (0, 1.5, 3, 3.5, 4, 4.5, and 5 µg/ml from CC dilutions and 0, 50, 100, 150, 200, 250, and 300 µg/ml from UDCA dilutions), as described in the previous step. After incubation, total RNA was extracted from the growth to measure gene expression in controls and with the effects of the drugs [25]. The primer sequences that provided by Sigma Aldrich Company\ Germany and used in this work for RT-PCR are listed in Table 1. This experiment was repeated three times.

2.5. Statistical analysis

An independent samples *t*-test was used to analyze the reported data. *P*-values < 0.05 were considered statistically significant. The statistical package for social sciences SPSS software program (version 24) was used to analyze the data.

3. Results

3.1. Effects of CC and UDCA on biofilm formation of *S. aureus* and *E. faecalis*

The *in vitro* study of the antibiofilm activity of CC and UDCA on both *S. aureus* and *E. faecalis* showed that a concentration of greater than 1.5 µg/ml of CC significantly ($p < 0.005$) inhibited the biofilm formation of bacterial isolates, and a concentration of greater than 50 µg/ml of UDCA significantly ($p < 0.005$) inhibited the biofilm formation of both bacterial isolates. However, the drugs had low effects ($p > 0.05$) on growth rates compared to the controls.

3.2. Effects of antibiofilm activity of CC and UDCA on expression of biofilm-related genes

The expression levels of biofilm genes in *S. aureus* and *E. faecalis* were studied under the effects of CC and UDCA in concentrations ranging from 1.5–5 µg/ml and 50–300 µg/ml, respectively. A dose-dependent decrease of the expression of the *icaR* and *icaA* genes occurred at these concentrations. However, expression of the *icaR* gene markedly decreased when *S. aureus* isolate was treated with CC at concentrations of 3–5 µg/ml and with all the tested concentrations of UDCA. In contrast, *icaA* gene expression strongly decreased at concentrations 3.5 and 4 µg/ml of CC and at 100 µg/ml of UDCA, compared to the control without drugs (Figures 1 and 3), and gradually increased with increasing concentrations of CC above 4 µg/ml and UDCA above 100 µg/ml (Figures 1 and 3). Inte-

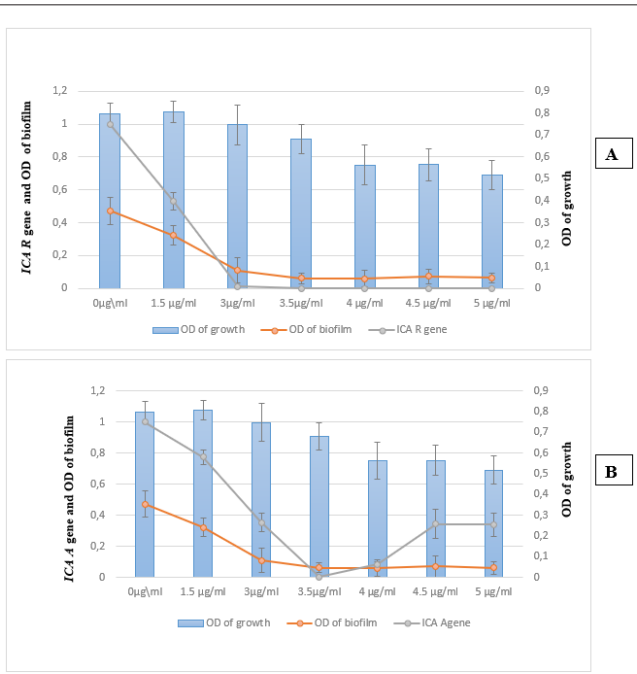

Fig. 1. Candesartan cilexetil (CC) significantly ($p < 0.005$) inhibited biofilm formation and expression level of *icaR* (A) and *icaA* (B) genes in *S. aureus* at concentrations 1.5 µg\ml and above with a low effect on growth rate. The data stands for the average of three independent experiments (mean ±SD).

Table 1. Primers used in RT-PCR to detect the biofilm formation-related genes in *S. aureus* and *E. faecalis*.

Genes	Sequences	Amplicon	References
16s rRNA	F: CTGGTAGTCCACGCCGTAAAC R: CAGGCGGAGTGCTTAATGC	90	[26]
ica R	F/ ATCTAATACGCCTGAGGA R/ TTCTTCCACTGCTCCAA	205	[27]
Ica A	F/ ACACTTGCTGGCGCAGTCAA R/ TCTGGAACCAACATCCAACA	188	[28]
rec A	F/CGACTAATGTCTCAAGCACTC R/CGAACATCACGCCAACTT	106	[29]
fsr c	F/GTGTTTTTGATTTCGCCAGAA R/TACGTTGTTCTTCCAAATAAGC	148	[30]
Gel E	F/ TACACCATTATCCAGAACT R/ CATCGCCATATTGAACTT	142	[29]

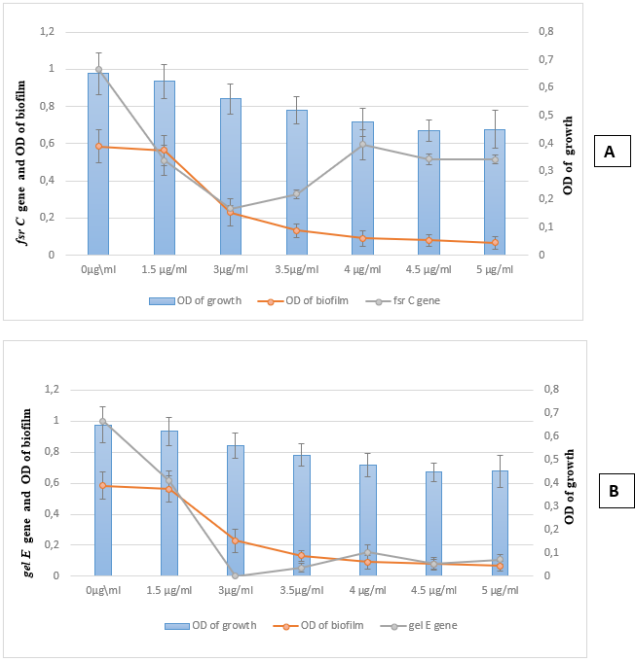

Fig. 2. Candesartan cilexetil (CC) significantly ($p < 0.005$) inhibited biofilm formation and expression level of *fsrC* (A) and *gelE* (B) genes in *E. faecalis* at concentrations 1.5 µg\ml and above with a low effect on growth rate. The data stands for the average of three independent experiments (mean ±SD).

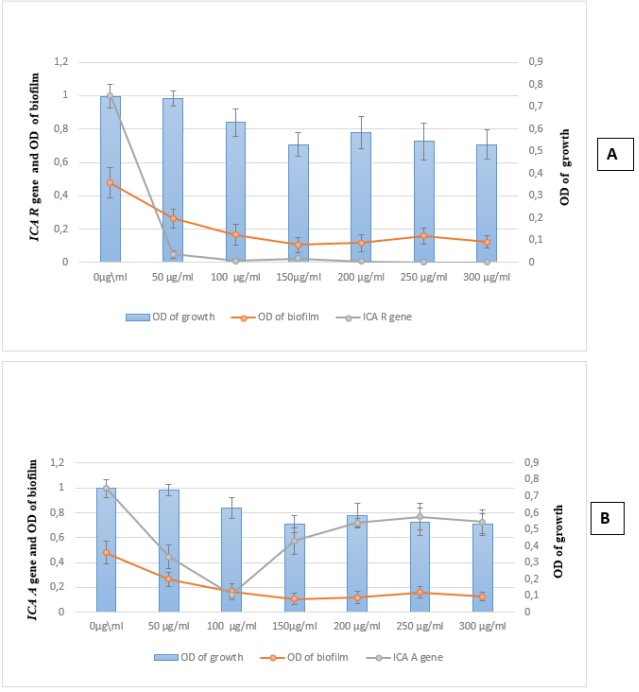

Fig. 3. Ursodeoxycholic acid(UDCA) siginficantly ($p < 0.005$) inhibited biofilm formation and expression level of *icaR* (A) and *icaA* (B) genes in *S. aureus* at concentrations 50 µg\ml and above with a low effect on growth rate. The data stands for the average of three independent experiments (mean ±SD).

restingly, similar results were observed when *E. faecalis* isolate was treated with the same concentrations of CC and UDCA. A strong reduction in *fsrC* gene expression occurred at concentrations of 3 µg/ml of CC and greater than 50 µg/ml of UDCA, while the expression of the *gelE* gene was markedly reduced at concentrations of 3–5 µg/ml of CC and greater than 100 µg/ml of UDCA (Figures 2 and 4).

4. Discussion

To date, the emergence and fast spread of multidrug-resistant bacteria have become major risks to health around the world, and, with the formation of biofilm, there has been an increase in the emergence of drug resistance. Traditional approaches, which attempted to reduce the spread of drug resistance by biofilm-forming bacteria, are no longer successful at the global level [31]. Therefore, to overcome these drawbacks, new anti-infective molecules should be developed, and the antibacterial activities of these new molecules must be examined against both planktonic bacteria and their biofilms. One extensively studied strategy is the use of non-antibiotic medications with antibiofilm and antimicrobial effects. CC is an angiotensin-receptor blocker. It is administered orally as the prodrug CC, which is rapidly converted to its active metabolite candesartan during absorption in the gastrointestinal tract. CC is used clinically for the treatment of hypertension and chronic heart failure, with a varying oral dose of 8–32 mg once a day [32]. Additionally, UDCA is a typical bile acid increasingly used for the management of chronic cholestatic liver diseases. It prevents the absorption and synthesis of cholesterol and can lead to the decomposition of gallstones. UDCA is commercially available as capsules and tablets with an oral dose of 300 mg two times a day for adults [33].

By using a CV staining assay, we found that CC and

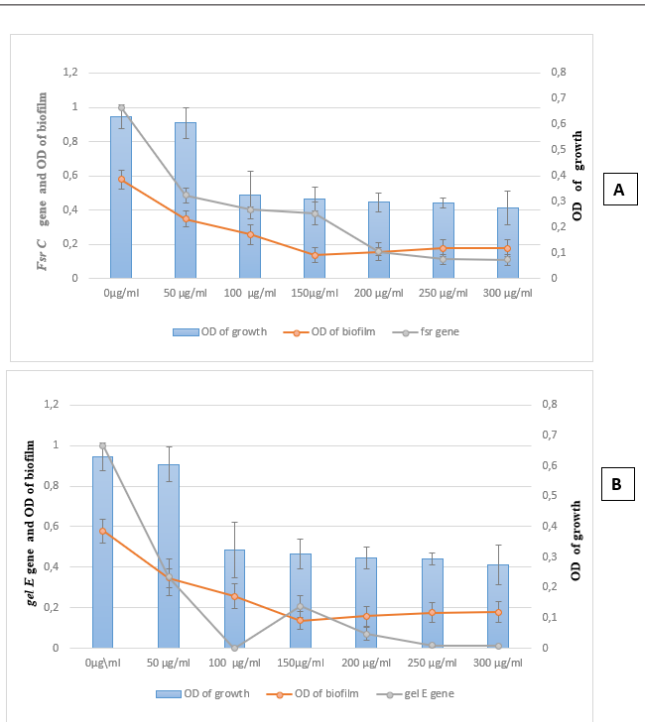

Fig. 4. Ursodeoxycholic acid(UDCA) significantly ($p < 0.005$) inhibited biofilm formation and expression level of *fsrC* (A) and *gelE* (B) genes in *E. faecalis* at concentrations 50 µg\ml and above with a low effect on growth rate. The data stands for the average of three independent experiments (mean ±SD).

UDCA effectively prevented biofilm formation in *S. aureus* and *E. faecalis*. Until now, the modes of action of CC and UDCA were unclear. However, according to the conclusions of Xu *et al.* [34], CC disrupted *Staphylococcus* membrane integrity, which caused noticeable leakage of intracellular contents. Considering that CC has an active

group present in other types of antimicrobial drugs, such as benzimidazole, we postulate that the toxicity of the drug causes stress mechanism by which it induces antibiofilm activity. He *et al*. [35] showed that UDCA treatment of a human colonic adenocarcinoma cell line (Caco-2) incubated with *Escherichia coli* directly blocked the growth and cell adherence of the bacteria. Mathai *et al*. [36] reported that 0.1% UDCA completely inhibited the growth of 16 of 27 clinical isolates of *Helicobacter pylori*, while inhibition of adherence to fetal intestinal cell lines occurred in only nine isolates. Additionally, another study indicated that UDCA inhibited entire vegetative cells and spores of *Clostridium difficile* in a dose-dependent manner [37].

To our knowledge, only one published study on the effect of CC on bacterial biofilm exists, in which authors Xu *et al.* [34] assessed the effect of CC on *S. aureus* biofilm, and their results were consistent with ours. Our study additionally delves deeper into the molecular regulation of biofilm genes, providing a more comprehensive understanding of how CC and UDCA reduced biofilm formation and mRNA expression levels in two infectious pathogens, *S. aureus* and *E. faecalis*. The tested doses of CC and UDCA in our study were lower than the human pharmaceutical daily doses. Therefore, we expected to observe almost no side effects, and no toxicity occurred.

In addition, our study indicated that the transcriptional levels of the *icaR* and *icaA* genes in *S. aureus* and the *fsrC* and *gelE* genes in *E. faecalis* were greatly decreased when these clinical isolates were treated with CC and UDCA, resulting in a notable reduction in biofilm formation by both isolates. Downregulation of *icaA* causes a decrease in the production of PIA and PNAG. This reduces biofilm formation because the *icaADBC* operon in *Staphylococcus* encodes enzymes necessary for the production of PIA and PNAG, both of which are crucial for the development of biofilms [38]. Increased transcription of the *icaA* gene at concentrations greater than 3.5 µg/ml of CC and greater than 100 µg/ml of UDCA may occur via a reduction in the expression of the regulatory gene *icaR*. This is because IcaR acts as a negative regulator of the *ica* operon, as Conlon *et al*. [9] indicated. *icaR* encodes a transcriptional repressor involved in environmental regulation of the expression of the *ica* operon and biofilm formation.

Our results showed a total suppression of both *icaR* and *icaA* expression at concentrations lower than those mentioned before (3.5 µg/ml of CC and 100 µg/ml of UDCA). This may be due to the environmental stress and potential toxicity caused by the drug, which may cause inactivation of the alternative transcription factor σ^B (a global regulator of the stress response in *S. aureus* and the expression of the *ica* operon). Indeed, Rachid *et al.* [18] concluded that inactivation of the alternative transcription factor σ^B resulted in a biofilm-negative phenotype. Suppression of both *icaA* and *icaR* simultaneously in this study indicated that regulation of *icaA* may be *icaR* independent at this time, as previously published data showed that the regulation of *ica* operon expression not only depends on the *icaR* regulator but is mediated by several regulatory factors, such as TcaR and SarA [8].

Suppression of *gelE* mRNA expression in *E. faecalis* when treated with concentrations greater than 3 µg/ml of CC and 100 µg/ml of UDCA may be related to the suppression of the fecal streptococci regulator fsr. This is because expression of the *gelE* gene is regulated tightly by the quorum-sensing system's fecal streptococci regulator locus *fsr* [39].

Our data showed an increase in *fsrC* gene expression but a decrease in *gelE* gene expression at concentrations greater than 4 µg/ml of CC. A possible reason for this may be that the *gelE* gene can be expressed independently of a functional *fsr* locus. As Hancock and Perego [39] indicated in their study, *gelE* was expressed independently of the *fsr* locus. However, the *fsr* locus consists of *fsrA, fsrB,* and *fsrC*, and the expression of the *gelE* gene is regulated by the entire locus, not only *fsrC* gene, and all three *fsr* genes are required for *fsr* function [40]. Given our findings and other previous results, we conclude that the *fsrC* and *gelE* genes are associated directly with biofilm formation in *E. faecalis*, and any decrease in the expression of these genes reduces biofilm formation.

5. Conclusion

CC and UDCA displayed potential antibiofilm effects against drug-resistant *S. aureus* and *E. faecalis*, by reducing the expression levels of biofilm related genes in these two bacterial isolates. This may cause less infection to occur in patients using these drugs, and the gelatinase enzyme may be a unique target for therapeutic intervention for enterococcal infection.

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Conflict of interests

The authors declare that they have no conflict of interest.

Consent for publications

The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study is approved by the ethical committee of the College of Medicine (Number 90).

Availability of data and material

All data generated during this study are included in this article.

Authors' contribution

The study concept was planned by Shwan Kamal Rachid; the methodology and writing the article by Bayan Taha Majid, Shwan Kamal Rachid, and Suha Ali Hussein.

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