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Research Article

Effect of combination of sub-inhibitory concentrations of cefepime and *Thymus vulgaris* essential oil in modulating biofilm formation of *Enterococcus faecalis in vitro*

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ABSTRACT

Modifying the biofilm-forming ability of *Enterococcus faecalis* may offer a valuable approach to treating infections. This study evaluated the effects of sub-minimum inhibitory concentrations (sub-MICs) of cefepime and *Thymus vulgaris* essential oil, alone and in combination, on the biofilm formation of *E. faecalis* isolated from urine samples of patients with urinary tract infections (UTIs). Minimum inhibitory concentrations (MICs) of both agents were determined, and their sub-MICs were tested using a polystyrene microtiter plate assay. Real-time PCR was used to assess the effect of cefepime sub-MICs on *esp* gene expression. The checkerboard assay was employed to evaluate the combined effect of both agents on biofilm inhibition. The incidence of UTI infection with *E. faecalis* was 13.15% of UTI cases. No significant correlation was observed between cefepime susceptibility and biofilm formation ($r = -0.28$, $P = 0.42$), whereas a significant positive correlation was found with *T. vulgaris* essential oil ($r = +0.66$, $P < 0.05$). Sub-MICs of cefepime (½, ¼, ⅛, and $\frac{1}{16}$ MIC) and *T. vulgaris* essential oil (½ and ¼ MIC) significantly reduced biofilm formation ($P < 0.05$). Additionally, cefepime sub-MICs significantly downregulated esp gene expression ($P < 0.05$). The combination of both agents at sub-MICs showed greater inhibition of biofilm formation than either agent alone. These findings suggest that cefepime and *T. vulgaris* essential oil, particularly in combination at sub-MIC levels, may be an effective strategy to control *E. faecalis* biofilm-associated infections.

Keywords:
Adhesion; Biofilm; Cefepime; *Enterococcus faecalis;* Sub-inhibitory MICs.

1. INTRODUCTION

The biofilm formation is a huge change in healing the infectious diseases caused by several pathogenic bacteria. The biofilm is a physical barrier that either prevent or reduce the antibiotics to reach the bacteria cells that impeded deeper in biofilm body.[1,2] The biofilm formed by different bacterial genera. *Enterococcus faecalis* is a Gram-positive streptococcal bacterium which concern in medical microbiology due to its resistance to wide spectrum of antibiotics by implementing different mechanisms one of them is the ability of this bacterial species to form biofilm on biotic and abiotic surfaces in vivo and in vitro.[3] It is a nosocomial pathogen and a part of the normal gut flora; however, it can cause severe endocarditis, urinary tract infection (UTI), and wound infections, particularly in immunocompromised patients.[4,5] The formation of biofilm in *E. faecalis* enhances bacteria to survive in hostile environments, contributes to persistent infections, and protects the bacteria from antibiotic therapy and host immune responses.[6]

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Cefepime is a fourth-generation cephalosporin, which is usually effective against a different pathogenic bacterial genus.[7] It is primarily used to treat urinary tract infections (UTIs) by binding penicillin-binding proteins (PBPs), modifying in the synthesis of bacterial cell wall that leading to limiting the bacteria growth.[8] *Thymus vulgaris* essential oil is extracted from the flowering parts and leaves of the *T. vulgaris*. It has been widely studied and used for its antimicrobial properties. It composed of several chemicals, i.g. thymol (20–60%) and carvacrol which have antimicrobial and antifungal properties.[9]

The sub-inhibitory concentrations [sub-minimum inhibitory concentrations (MIC)] influence bacterial behavior, including biofilm formation, without inhibiting bacterial growth. Previous studies showed that the sub-MICs of antibiotics promote biofilm-related genes or facilitating adhesion to different surfaces.[10] Recent studies showed that the sub-MICs of antibiotics decrease the biofilm production to biotic and abiotic surfaces.[11-13] Biofilm formation enhances pathogenicity and increases bacterial resistance to antibiotic treatment.[6] Previous studies reported the involving the Enterococcal Surface Protein (*esp*) gene in the biofilm formation of *E. faecalis* including.[14] and the other bacteria[15]

Understanding the effect of cefepime and *T. vulgaris* essential oil sub-MICs, and the combination of sub-MICs of both agents on biofilm formation is important for establishing the future strategies of public health programs, as it may provide insight into novel treatment strategies and inform clinical practice. The above points that would be clear in the present study are scanty in literature that is why the present study aims to fill the gap of the knowledge regarding the level of effects of cefepime and *T. vulgaris* essential oil sub-MICs on the biofilm formation and the possibility of use the combination of these agents in improving the antibacterial effect of the cefepime against *E. faecalis.*

2. MATERIALS AND METHODS

2.1 Isolation and Identification

The midstream urine specimens were collected in a sterile container from inpatients (73 samples) suffering from UTIs (Baghdad Teaching Hospital, Baghdad, Iraq). All patients did not receive antibiotic treatment 72 h before the sample collection date and consented to participate in the study (They signed the consent form). The samples were immediately transferred to the laboratory. Briefly, the urine samples were cultured onto MacConkey agar (HiMedia, Mumbai, India), and cysteine lactose electrolyte deficient (CLED) (Himedia) and incubated for 18 h at 37 ºC. The bacteria were identified based on appearance on gram stain, growth in 6.5% NaCI, catalase-negative, and growth on bile esculin medium (Hardy Diagnostics). The VITEK 2 DensiCheck instrument (bio- Mérieux, Marcy-l'Étoile, France) was used to confirm the identification of the isolates.[16] The bacterial isolates were subcultured onto the nutrient agar plates for short-term storage.[17]

2.2 *T. vulgaris* essential oil

The standard method of Galovičová et a., (2021) were followed to prepare *T. vulgaris* essential oils. The plant samples (flower) were purchased from the local market. The plants were cleaned, then washed by distilled water and dried at room temperature, and then storage in clean conditions until use. The essential oils of these plants were prepared by dried the plant parts. Two hundred fifty grams of dried parts by steam distillation method (Cleavenger) using DMSO (Dimethyl Sulfoxide). The prepared essential oils were dried at 37 ºC and kept at 4º C until used.[18]

2.3 Minimum inhibitory concentration (MIC)

The micro-dilution method of Al-Mutalib & Zgair (2023) was followed to measure the MICs of cefepime and *T. vulgaris* essential oil against ten *E. faecalis* (Ef1, Ef2, Ef3, Ef4, Ef5, Ef7, Ef8, Ef9, Ef10, and Ef18) isolates. 500 µg of cefepime (Sanofi) was dissolved in sterile Mueller–Hinton broth to prepare a stock concentration (2 mg/ml). Double-fold dilutions (100 µL) were prepared in the microtiter plate (U shape) with sterile MHB (HiMedia). Five microliters of *E. faecalis* were added to each well. The bacterial suspension was prepared by washing the overnight growth (grown at 37 ºC in MHB) of bacteria with sterile phosphate-buffered saline (PBS; 0.1 M, pH 7.2) using centrifugation at 5,000 g for 10 min (Beckman Coulter, Brea, USA), and the optical density of bacterial suspension was adjusted to 0.1 at the wavelength 600 nm (double-beam spectrophotometer model SP-MUV8000T; Bioevopeak, Jinan, China). Similar method was followed to evaluate the MIC of *T. vulgaris* essential oil against the ten *E. faecalis.* The plates were shaken gently and incubated at 37 ºC for 18 h. Control 1: wells contain MHB and bacterial isolate, control 2: wells contain MHB only. Control 3: different double dilutions of anti-bacterial agents (cefepime or *T. vulgaris* essential oil) in MHB. The lowest antibiotic concentrations completely inhibit growth considered MIC.[11]

2.4 Biofilm formation

The previous method was followed to measure the biofilm formation of the ten *E. faecalis* isolates. Briefly, 200 µl of sterile Tryptic Soy Broth (TSB, HiMedia) were added to flat-bottom polystyrene tissue culture plate wells. The standard inoculum (5 µl, 0.1 at

600 nm) of *E. faecalis* (described in 2.3) was added to each well and incubated at 37 ºC for 24 h. The TSB was discarded, and the plates were washed three times with sterile distilled water. The plates were dried and 200 µl of Hucker crystal violet (0.4%) was added to each well. The plates were incubated at room temperature for 15 min and washed 5 times with distilled water. After drying the wells, 200 µL of anhydrous ethanol was added to the wells. The optical density (OD) was measured at a wavelength of 570 nm using a microplate reader (BioTek 800 TS; Winooski, USA). The experiment was repeated in triplicate.[11-13,19]

2.5 Effect of sub-MICs on biofilm formation

In this experiment, the effect of different concentrations of cefepime and *T. vulgaris* essential oil on the biofilm formation of *E. faecalis* isolate that produced the level of biofilm. The previous method was followed to evaluate the level of biofilm formation with modifications.[12,13] Instead of TSB, the serial dilutions of sub-MICs of cefepime MICs [½ MIC (31.25µg/mL), ¼ MIC (15.62 µg/mL), $^1/_8$ MIC (7.8 µg/mL), $^1/_{16}$ MIC (3.9 µg/mL), $^1/_{32}$ MIC (1.9 µg/mL), and $^1/_{64}$ MIC (0.97 µg/mL)] and serial dilution of *T. vulgaris* essential oil presented in dilutions [½ MIC (0.0125), ¼ MIC (0.00625), $^1/_8$ MIC (0.003125), $^1/_{16}$ MIC (0.001563), $^1/_{32}$ MIC (0.000781), and $^1/_{64}$ MIC (0.000391)] were prepared in TSB (Himedia) in the wells of a polystyrene microtiter plate (flat shape) were used. The plates were incubated at 37 °C for 18 h and then washed three times with sterile distilled water to remove non-adherent bacteria. The wells were subsequently air-dried. Then, 200 µL of crystal violet solution was added to each well and incubated at room temperature for 15 min. After staining, the wells were washed five times with distilled water to remove excess dye and were then allowed to dry. Two hundred microliters of anhydrous ethanol were added to each well. The absorbency was measured (OD $^{570\ nm}$) using a microplate reader (BioTek 800 TS, Winooski, USA). The plate count method was used to check the viable number of bacteria under the stress of sub-MICs (cefepime and T. vulgaris essential oil) before starting the experiment of evaluation the biofilm formation. The experiment was repeated in triplicate.[12,13]

2.6 *Esp* gene detection and expression

The standard previous method was followed to detect the *esp* gene in the *E. faecalis* that produced the highest level of biofilm, using the Thermal Cycler machine model T100 (Bio-Rad Lab. USA), the sequence of primer and the conditions of experiment was describe in details in previous studies.[20,14] The RNA was isolated from bacterial suspension (10^9 c.f.u./ml) using the previous procedures described by Cheung et al. (1994).[21] The RNA purification and cDNA generation were done as described by Nallapareddy et al. (2006).[22] The TaqMan Universal PCR master mix (Applied Biosystems) was used to do the Real-time PCR (7500 Fast Real-Time PCR System, Applied Biosystems™, USA). The condition of real-time procedure was descripted previously.[14] The $\Delta\Delta CT$ method of Livak et al. (2001) was used to calculate the difference in *esp* mRNA present in bacteria grown at 37°C using 23S rRNA as an internal control.[23] In this experiment the expression of gene was measured in the Ef9 under the stress of antibiotic and compared with the expression of the same gene in Ef9 without any stress. Analyses were performed in triplicate.

2.7 Effect of combination of sub-MICs of H_2O_2 and cefotaxime on biofilm formation

The checkerboard assay in a 96-well (flat-shape) polystyrene microtiter plate was used to determine whether the combination of cefepime and *T. vulgaris* essential oil enhances antibiofilm efficacy compared to either material alone. In the study the *E. faecalis* that produced the highest biofilm production was used. Briefly, 100 µL of sterile TSB (Hi-Media, India) was added to each well. A horizontal double-fold serial dilution of cefepime (Sanofi) was prepared in double fold dilutions (from 2000 to 1.95 µg/ml) and applied across all wells in each row of the plate (wells 1 to 11). A vertical double-fold serial dilution of *T. vulgaris* essential oil (from $^1/_{160}$ to $^1/_{10240}$, stock solution prepared freshly) was prepared starting from row A to G of microtiter plate. Subsequently, 5 µL of *E. faecalis* (optical density 0.1 at 600 nm, prepared according to the method describes in 2.3) was added to each well. The microtiter plates were gently shaken and incubated at 37 ºC for 18 h. Then, the plates were dried and stained with 100 µL of Hucker crystal violet (0.4%) for 15 minutes, then washed five times with distilled water. After drying, 100 µL of anhydrous ethanol was added to each well. The absorbance was measured at 570 nm using a microplate reader (BioTek 800 TS; BioTek, Winooski, USA). The experiment was repeated in triplicate.[11,24,25]

2.8 Statistical analyses

The statistical analysis was conducted and the graphs were generated utilizing Origin v. 8 software (OriginLab, Northampton, USA). The data were presented as means ± standard error (M ± SE). The disparities were assessed utilizing a student's t-test and one-way analysis of variance (ANOVA). The relationship was evaluated with Pearson's correlation coefficient (r). A $P< 0.05$ was deemed statistically significant.[26, 27]

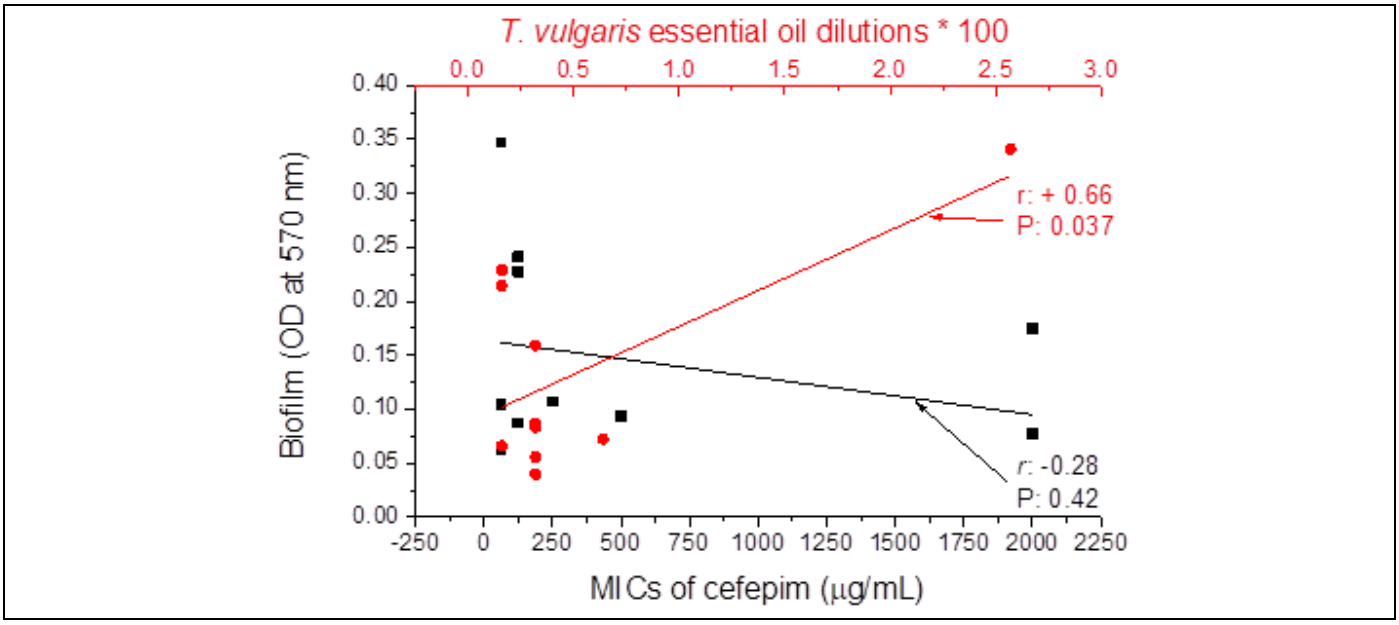

Figure 1. The relationship between biofilm formation of the ten isolates of *E. faecalis* and MICs of cefepime (black line and dots) and *T. vulgaris* essential oil (red line and dots). *r*: Pearson correlation coefficient; P<0.05 considered a significant relationship. OD, optical density.

3. RESULTS

3.1 Bacterial isolates

Ten isolates of *E. faecalis* were isolated from 73 urine samples collected aseptically from patients suffering from UTIs. The bacterial species were identified using microscopic and biochemical tests. The isolates were further confirmed as *E. faecalis* using VITEK 2 technology. The present study revealed a high incidence of urinary tract infections with *E. faecalis* (13.15%).

3.2 Antibiotic susceptibility and biofilm formation

The MICs were measured for ten isolates of *E. faecalis* against cefepime. The results showed that the highest MIC of cefepime was against Ef3 and Ef18 (2000 µg/ml) followed by Ef10, while the lowest MIC of cefepime was against Ef7, Ef8, and Ef 9 (62.5 µg/ml) . The highest *T. vulgaris* MIC dilutions were observed against Ef1, Ef2, and Ef4, while Ef9 exhibited the lowest dilution. The *E. faecalis* (Ef9) produced the highest level of biofilm (0.34 ± 0.06) followed by Ef4 (0.24 ± 0.096). The lowest biofilm was produced by Ef8 (0.062 ± 0.015), Table 1. The study showed that the isolates produced the biofilm on a polystyrene microtiter plate. That is why the isolate Ef9 will be used in further experiments.

The relationship between the biofilm formation of the ten isolates and the MICs of cefepime and *T. vulgaris* essential oil against ten isolates of *E. faecalis* that were isolated from urine specimens collected from patients with UTI (Figure 1). The study showed there is no statistical correlation between the MIC of cefepime (*r:* -0.28, P>0.05) against ten isolates and the biofilm formation of the same isolates. However, statistical correlation was seen between the MIC of *T. vulgaris* essential oil (*r*: +0.66, P<0.05) against ten isolates and the biofilm formation of the same isolates.

3.3 Effect of Sub-MICs of agents on biofilm formation

Effects of the sub-inhibitory concentrations of cefepime and *T. vulgaris* essential oil on the biofilm

Table 1. The minimum inhibitory concentrations of cefepime and *T. vulgaris* essential oil against ten isolates of *E. faecalis* and biofilm formation of the same isolates. The results presented in mean ± standard deviation (SD). OD, optical density

No	Isolates	Cefepime MIC (µg/mL)	*T. vulgaris* essential oil MIC (dilutions)	Biofilm $OD^{600\ nm}$
1	Ef 1	31.2 ± 31.2	0.00161 ± 0.00033	0.22775 ± 0.0818
2	Ef 2	125 ± 15.6	0.00161 ± 0.00033	0.0875 ± 0.03254
3	Ef 3	2000 ± 31.2	0.0032 ± 0.00065	0.0775 ± 0.04162
4	Ef 4	125 ± 15.6	0.00162 ± 0.00031	0.24125 ± 0.096
5	Ef 5	250 ± 15.6	0.0032 ± 0.00065	0.107 ± 0.04546
6	Ef 7	62.5 ± 62.5	0.0032 ± 0.00065	0.10375 ± 0.03796
7	Ef 8	62.5 ± 31.2	0.0032 ± 0.00065	0.0625 ± 0.01563
8	Ef 9	62.5 ± 125.6	0.025694 ± 0.005243	0.34725 ± 0.06999
9	Ef 10	500 ± 15.6	0.0064 ± 0.0013	0.0935 ± 0.04847
10	Ef 18	2000 ± 250	0.0032 ± 0.00065	0.175 ± 0.03385

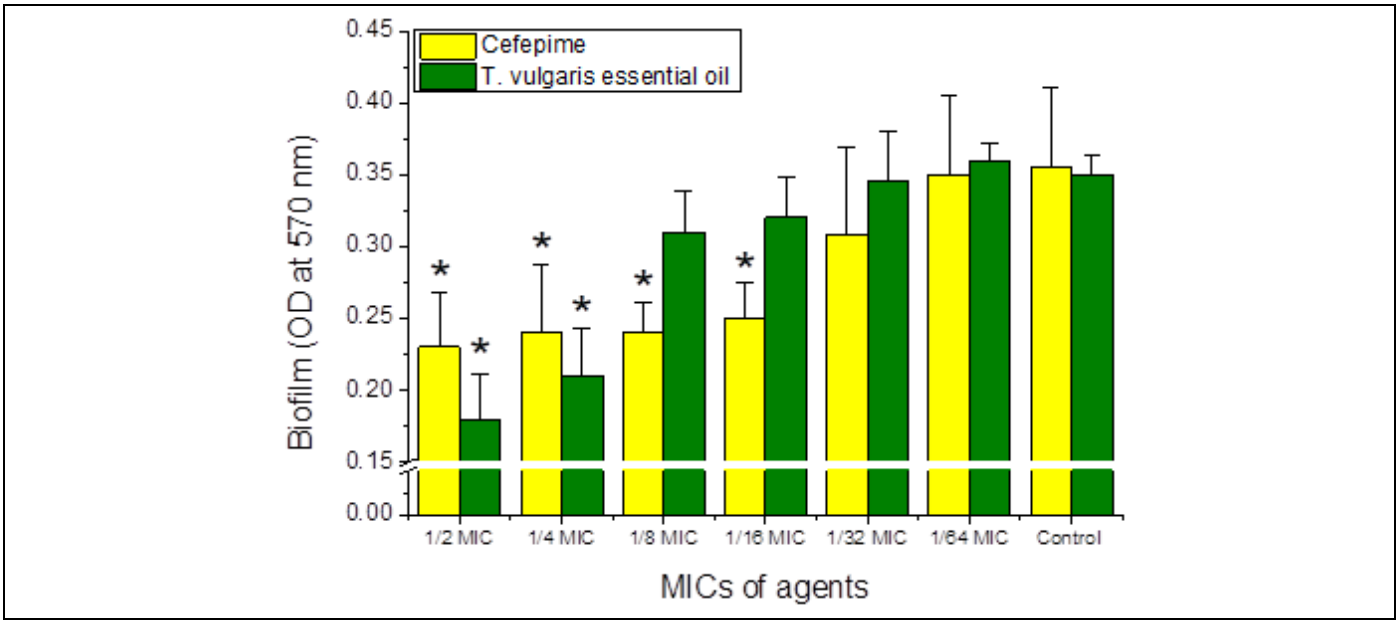

Figure 2. Effect of cefepime sub-MICs [½ MIC (31.25µg/mL), ¼ MIC (15.62 µg/mL), $^{1}/_{8}$ MIC (7.8 µg/mL), $^{1}/_{16}$ MIC (3.9 µg/mL), $^{1}/_{32}$ MIC (1.9 µg/mL), and $^{1}/_{64}$ MIC (0.97 µg/mL)] and *T. vulgaris* essential oil sub-MICs presented in dilutions [½ MIC (0.0125), ¼ MIC (0.00625), $^{1}/_{8}$ MIC (0.003125), $^{1}/_{16}$ MIC (0.001563), $^{1}/_{32}$ MIC (0.000781), and $^{1}/_{64}$ MIC (0.000391)] on biofilm formation of *E. faecalis* isolate that produced the highest level of biofilm (Ef 9). Results were expressed in the average biofilm formation of Ef9 with standard deviation [mean ± SD (standard deviation)]. Asterisks indicate a significant difference (P<0.05) from the control (biofilm level according to optical density (OD) values at 570 nm without agents' stress). The results presented in mean ± standard deviation (SD).

formation of the *E. faecalis* (Ef 9) isolate that produced the highest level of biofilm to polystyrene were evaluated (Figure 2). The results were expressed as the mean ± standard deviation of the biofilm formation (OD^{570nm}). The current study demonstrated that sub-MICs of cefepime (½ MIC, ¼ MIC, $^{1}/_{8}$ MIC, and $^{1}/_{16}$ MIC) significantly reduced biofilm production ($P < 0.05$) compared to the control (biofilm formation of *E. faecalis* without exposure to cefepime). The treatment of the isolates with $^{1}/_{32}$ and $^{1}/_{64}$ of cefepime did not significantly affect (P>0.05) the biofilm formation to polystyrene. The present study showed that sub-MICs of (Ef9), which produced the highest level of biofilm on the polystyrene microtiter plates. Figure 3 shows that sub-MICs of cefepime (½ MIC, ¼ MIC, $^{1}/_{8}$ MIC, $^{1}/_{16}$ MIC) significantly down-regulated the *esp* gene expression (P<0.05). However, no significant change in *esp* gene expression was observed when the Ef9 was pre-exposed to $^{1}/_{32}$, and $^{1}/_{64}$ MICs of cefepime (P>0.05).

3.5 Effect of combination of Agents on Biofilm Formation

The present study demonstrated the effect of combining different concentrations of cefepime with various dilutions of *T. vulgaris* essential oil on the biofilm formation of the Ef9 isolate. The results indicated that the combination of both agents significantly reduced biofilm formation ($P < 0.05$) compared to the effect of each agent alone (control 1 and control 2). The greatest reduction in biofilm formation *T. vulgaris* essential oil up to ¼ reduced the biofilm formation (P<0.05), However, the other sub-MICs of *T. vulgaris* essential oil ($^{1}/_{8}$, $^{1}/_{16}$, $^{1}/_{32}$, and $^{1}/_{64}$ MIC) did not reduced the biofilm formation to polystyrene microtiter plate significantly (P>0.05).

3.4 Effect of sub-mics of cefepime on *esp* gene expression

The effect of sub-MICs of cefepime on *esp* gene expression was shown in the present study. The expression of this gene was investigated in *E. faecalis* was observed with the combination of ½ MIC of both agents, resulting in an optical density (OD) of 0.9 ($P < 0.05$ compared to control 1 and control 2). In contrast, the lowest reduction was observed with the combination of $^{1}/_{64}$ MIC of both agents, yielding an OD of 0.29, which was not statistically significant ($P > 0.05$ compared to control 1 and control 2), Table 2.

4. DISCUSSION

The biofilm is a significant factor contributing to persistence and virulence of pathogenic bacteria in different places either in vivo or invitro, especially in nosocomial acquired infections that exhibit resistance of bacteria to a wide spectrum of antibiotics. Previous studies have shown that sub-inhibitory concentrations of antibiotics variably affect bacterial biofilm formation.[11-13,20,28,29] Investigating the effect of cefepime concentrations in alteration of *E. faecalis* biofilms may

Table 2. The biofilm formation of *E. faecalis* (Ef9) under the stress of different concentrations of cefepime and dilutions of *T. vulgaris* essential oil in terms of optical density at 570 nm. Cintrol1: effect of different concentrations of cefepime on the biofilm formation of Ef9; control 2: effect of different dilution of *T. vulgaris* essential oil on the biofilm formation; control 3: biofilm formation of Ef9 without any stress.

T. vulgaris essential oil dilutions	Concentrations of cefepime in microgram/ml (µg/ml)												
	2000	1000	500	250	125	*62.5 MC*	31.25	15.6	7.8	3.9	1.95	0.97	control 2 (0 Cefepime)
0.1	0.038	0.039	0.036	0.035	0.038	*0.036*	0.037	0.04	0.039	0.034	0.041	0.04	0.04
0.05	0.04	0.041	0.04	0.038	0.039	*0.041*	0.04	0.039	0.037	0.039	0.039	0.038	0.035
0.025 (MC)	*0.04*	*0.039*	*0.041*	*0.038*	*0.041*	*0.042*	*0.039*	*0.042*	*0.038*	*0.04*	*0.039*	*0.04*	*0.037*
0.012 ½ MC	0.04	0.042	0.044	0.05	0.046	*0.045*	0.09	0.11	0.13	0.13	0.12	0.15	0.18
0.0062 ¼ MC	0.04	0.04	0.04	0.046	0.047	*0.0441*	0.1	0.13	0.15	0.159	0.18	0.19	0.21
0.00313 1/8 MC	0.039	0.04	0.044	0.045	0.043	*0.0411*	0.14	0.13	0.16	0.19	0.26	0.257	0.31
0.00156 1/16 MC	0.037	0.04	0.041	0.042	0.045	*0.052*	0.12	0.135	0.17	0.21	0.256	0.26	0.32
0.000782 1/32 MC	0.0384	0.039	0.042	0.039	0.049	*0.05*	0.14	0.17	0.203	0.198	0.257	0.27	0.345
0.00039 1/64 MC	0.041	0.042	0.043	0.044	0.051	*0.046*	0.183	0.183	0.19	0.18	0.234	0.29	0.36
0 (control 1)	0.041	0.039	0.05	0.04	0.042	*0.049*	0.22	0.24	0.24	0.25	0.308	0.35	0.355 (control 3)

provide appreciated insights into developing more effective treatment strategies and the effect of low concentrations of antibiotics in curing *E. faecalis* infectious diseases including UTIs.[30] Previous studies showed that the resistance to the antibiotics increase time to time, thus the using of safe materials as the alteration strategies in the treatment of the infectious diseases cause by the high resistance bacteria to antibiotic is considered a promising treatment strategy. Several previous studies reported the antimicrobial and anti-biofilm effect of *T. vulgaris* essential oil.[31-33]

In the current study, ten isolates of *E. faecalis* were investigated. A relatively high incidence of infection was associated with these isolates. The MICs of cefepime and *T. vulgaris* essential oil were determined to assess their antimicrobial activity. The isolates exhibited variable susceptibility to both agents. Similarly, biofilm formation capacity varied among the isolates. No significant correlation was observed between biofilm formation and susceptibility to cefepime. This improvement suggests that, in addition to biofilm formation, other mechanisms may contribute to the response of *E. faecalis* to cefepime i.e. low binding affinity of penicillin-binding proteins (PBPs) and *E. faecalis* strains produce β-lactamases that hydrolyze β-lactam antibiotics.[34,35] In contrast, a positive correlation was found between biofilm formation and the dilution of *T. vulgaris* essential oil. Sub-inhibitory concentrations of both cefepime and *T. vulgaris* essential oil reduced biofilm formation on abiotic surfaces (polystyrene) to varying degrees. Moreover, higher sub-MICs of cefepime were found to downregulate the expression of the *esp* gene. The combination of different concentrations of cefepime and dilutions of *T. vulgaris* essential oil resulted in a greater reduction in biofilm formation compared to the effect of each agent alone

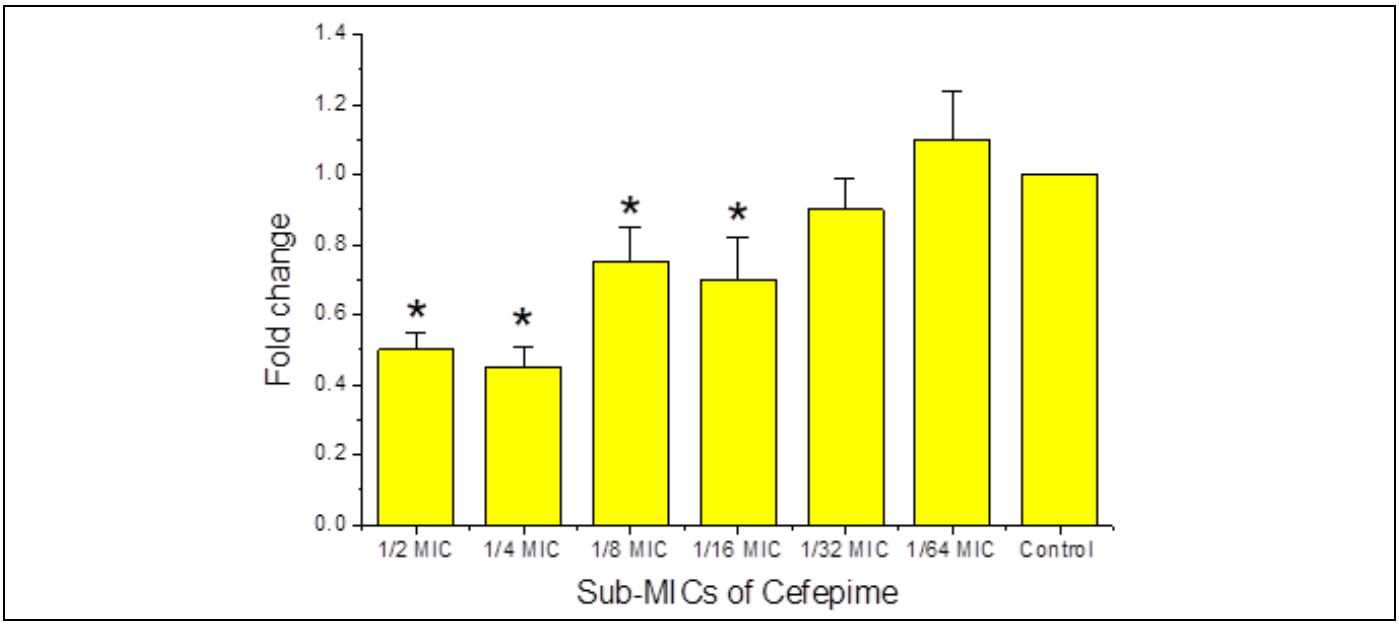

Figure 3. Fold change of *esp* gene expression of *E. faecalis* (Ef9) post-exposure to the different concentrations of sub-MICs of cefepime. Asterisks indicate a significant difference (P<0.05) from the control (Fold change of *esp* gene expression of *E. faecalis* without cefepime stress). The results presented in mean ± standard deviation (SD).

No previous studies investigated the effect of sub-inhibitory concentrations of cefepime. The previous study of Liu et al., (2020) highlighted the effect of sub-MIC of *T. vulgaris* essential oil on the biofilm formation of *E. faecalis*.[36] But there is no previous study investigated the effect of the combination of both agents on the biofilm formation of *E. faecalis*. Previous study investigated the sub-MICs of ampicillin, penicillin, and gentamicin interfere with the expression of biofilm formation genes of *E. faecalis* and they suggested this topic required further study to clarify the role of sub-MIC of the antibiotics on the biofilm formation of *E. faecalis*.[20] Another study reported that the sub-MICs of penicillin G, amoxicillin, doxycycline, fosfomycin, tetracycline, and vancomycin enhanced the biofilm formation of *E. faecalis*.[37] However, earlier studies showed that the sub-MICs of ofloxacin, rifampicin, and ceftriaxone decreased the biofilm formation of *P. aeruginosa in vitro*.[11-13] Previous study showed that the sub-MIC of *T. vulgaris* essential oil reduced the biofilm formation of candid albicans and the study also showed the synergistic effect of sub-MICs of *T. vulgaris* essential oil with antifungal medicine.

The decreasing in biofilm formation of bacteria observed in this study may be attributed to the interference of antibiotics with key mechanisms involved in biofilm formation, such as environmental conditions, quorum sensing (QS), gelatinase (GelE), cytolysin, enterococcal surface protein (Esp), and the role of bacterial appendages.[20, 24, 38-42].The outcome of the present study showed that the combination of sub-MICs of cefepime and *T. vulgaris* essential oil reduce the biofilm formation, suggesting their potential as adjunct therapies to combat biofilm-associated infections and improve treatment outcomes in UTI patients. Further research is necessary to clarify the mechanisms of sub-MIC levels to reduce biofilm formation and to assess their impact on bacterial susceptibility to antibiotics. While our study provides valuable insights, reliance solely on *in vitro* results is insufficient; *in vivo* studies are essential to validate and extend these findings. The current study opens the new approach of possibility of using the safe material of *T. vulgaris* essential oil in improving the activity of the antibiotics.

5. CONCLUSION

The present study revealed a relatively high incidence of urinary tract infections (UTIs) caused by *E. faecalis*. The ability of *E. faecalis* to form biofilms and its susceptibility to cefepime and *T. vulgaris* essential oil varied among isolates. No significant correlation was found between biofilm formation and susceptibility to cefepime. However, a significant positive correlation was observed between biofilm formation and the different dilutions of *T. vulgaris* essential oil. Sub-MICs of both cefepime and *T. vulgaris* essential oil reduced biofilm formation by *E. faecalis* in a concentration-dependent manner. Furthermore, sub-MICs of cefepime downregulated the expression of the *esp* gene, providing insights into the potential mechanism through which cefepime inhibits biofilm formation. The combination of cefepime and *T. vulgaris* essential oil significantly reduced biofilm formation, suggesting that using both agents at sub-MIC levels may serve as a promising strategy for treatment.

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Author contribution

Hwazen Amer Shnyoor: Writing – original draft, Methodology, Investigation, Formal analysis.
Ayaid Khadem Zgair**:** Writing – review & editing, Validation, Methodology, Formal analysis, Supervision, Conceptualization.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval

The Human Ethical Committee of the Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq, approved the study and provided the official signed approval letter with Reference No. CSEC/1025/0091 on May 8, 2024.

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REFERENCES

1. Ciarolla AA, Lapin N, Williams D, Chopra R, Greenberg DE. Physical approaches to prevent and treat bacterial biofilm. Antibiotics (Basel). 2022;12(1):54.
2. Mohammed NS, Obaid MM, Jasem MA, Noaman TM. Impact of biofilm formation on antibiotic resistance in *Escherichia coli*. World J Exp Biosci. 2024; 12(2): 44-8.
3. Mahood WS, Majeed AA. Transformation and over expression of chromosomal bile salt hydrolase gene A (bshA) that transfer from transformer *Escherichia coli* to *Enterococcus faecalis*. World J Exp Biosci. 2016; 4(1): 70-4.

4. Sangiorgio G, Calvo M, Migliorisi G, Campanile F, Stefani S. The impact of *Enterococcus* spp. in the immunocompromised host: A comprehensive review. Pathogens. 2024;13(5):409.
5. Kim YB, Seo KW, Shim JB, Son SH, Noh EB, Lee YJ. Molecular characterization of antimicrobial-resistant Enterococcus faecalis and *Enterococcus faecium* isolated from layer parent stock. Poult Sci. 2019;98(11):5892-9.
6. Khalil MA, Alorabi JA, Al-Otaibi LM, Ali SS, Elsilk SE. Antibiotic resistance and biofilm formation in *Enterococcus* spp. isolated from urinary tract infections. Pathogens. 2022;12(1):34.
7. Okamoto MP, Nakahiro RK, Chin A, Bedikian A, Gill MA. Cefepime: a new fourth-generation cephalosporin. Am. J. Hosp. Pharm. 1994;51(4):463-77.
8. Zhanel GG, Mansour C, Mikolayanko S, Lawrence CK, Zelenitsky S, et al. Cefepime-taniborbactam: A novel cephalosporin/β-lactamase inhibitor combination. drugs. 2024;84(10):1219-50.
9. Kachur K, Suntres Z. The antibacterial properties of phenolic isomers, carvacrol and thymol. Crit Rev Food Sci Nutr. 2020;60(18):3042-53.
10. Dawoud AM, Saied SA, Malek MM, Sabal MS. Impact of sub-inhibitory antibiotic concentrations on biofilm formation among nosocomial isolates of *Enterococcus* species. Microbes Infect. Dis. 2024;5(2):663-75.
11. Al-Mutalib LAA, Zgair AK. Effect of subinhibitory doses of rifaximin on in vitro *Pseudomonas aeruginosa* adherence and biofilm formation to biotic and abiotic surface models. Polim. Med. 2023;53(2): 97-103.
12. Al-Mutalib LAA, Zgair AK. Sub-inhibitory doses of Ofloxacin reduce adhesion and biofilm formation of *Pseudomonas aeruginosa* to biotic and abiotic surfaces. Pharm. Sci. Asia. 2023; 50(3): 196-203.
13. Talib MM, Ghafil JA. Effect of sub-minimum inhibitory concentrations of ceftriaxone on the *Pseudomonas aeruginosa* adhesion to human oral mucosal epithelial cells and biofilm formation to polystyrene in vitro. Pharm. Sci. Asia. 2024;51(2):180-9.
14. Van Wamel WJ, Hendrickx AP, Bonten MJ, Top J, Posthuma G, Willems RJ. Growth condition-dependent Esp expression by *Enterococcus faecium* affects initial adherence and biofilm formation. Infect Immun. 2007;75(2):924-31.
15. Sugimoto S, Iwamoto T, Takada K, Okuda K, Tajima A, Iwase T, Mizunoe Y. Staphylococcus epidermidis Esp degrades specific proteins associated with Staphylococcus aureus biofilm formation and host-pathogen interaction. J Bacteriol. 2013 Apr;195(8):1645-55. doi: 10.1128/JB.01672-12.
16. Parameswarappa J, Basavaraj VP, Basavaraj CM. Isolation, identification, and antibiogram of enterococci isolated from patients with urinary tract infection. Ann. Afr. Med. 2013;12:176-81.
17. Zgair AK, Jabbar H, Ghafil JA. Biosorption of Pb and Ni from aqueous solution by *staphylococcus aureus*, *Pantoea* and *Pseudomonas aeruginosa*. Iraqi J. Sci. 2019;60(4):739-44.
18. Galovičová L, Borotová P, Valková V, Vukovic NL, Vukic M, Štefániková J, Ďúranová H, Kowalczewski PŁ, Čmiková N, Kačániová M. *Thymus vulgaris* Essential Oil and Its Biological Activity. Plants (Basel). 2021;10(9):1959.
19. Ghafil JA, İbrahim BMS, Zgair AK. Coating indwelling urinary catheters with moxifloxacin prevents biofilm formation by *Burkholderia cepacia*. Polim Med. 2022 Jan-Jun;52(1):7-11. doi: 10.17219/pim/149986.
20. Caixeta Magalhães Tibúrcio AA, Paiva AD, Pedrosa AL, Rodrigues WF, Bernardes da Silva R, Oliveira AG. Effect of sub-inhibitory concentrations of antibiotics on biofilm formation and expression of virulence genes in penicillin-resistant, ampicillin-susceptible *Enterococcus faecalis*. Heliyon. 2022;8(10):e11154.
21. Cheung AL, Eberhardt KJ, Fischetti VA. A method to isolate RNA from gram-positive bacteria and mycobacteria. Anal. Biochem.1994;222:511-514.
22. Nallapareddy SR, Murray BE. Ligand-signaled upregulation of *Enterococcus faecalis* ace transcription, a mechanism for modulating host-E. faecalis interaction. Infect. Immun.2006;74:4982-4989.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 2001;25:402-408.
24. Mohammed HA, Zgair AK. Detection of quorum sensing genes of *Pseudomonas aeruginosa* isolated from different areas in Iraq. Iraqi J. Sci. 2022;63(11):4665-4673.
25. Zgair AK, Chhibber S. Adhering ability of *Stenotrophomonas maltophilia* is dependent on growth conditions. Mikrobiologiia. 2011 Jul-Aug;80(4):459-64.
26. Zgair AK. Flagellin administration protects respiratory tract from *Burkholderia cepacia* infection. J. Microbiol. Biotechnol. 2012;22(7):907-16.
27. Ghafil JA, Zgair AK. Bacterial secretions in growth medium stimulate the mouse respiratory innate immune response. J. Med. Microbiol. 2022;71(10).
28. Ghafil JA, Al-Sudani KSF, Ibrahim Salih BM, Alshahrani AM, Zgair AK. Relationship between biofilm formation of *Pseudomonas aeruginosa* and susceptibility to rifaximin and ofloxacin. Pak J Pharm Sci. 2024 Nov-Dec;37(6):1609-1614.
29. Zgair AK, Ghafil JA, Radif HM, Radhi SN, Hafiz MH, Albaayit SFA. Moxifloxacin reduces *Stenotrophomonas maltophilia* adhesion to mouse intestinal tract in vitro. Pak J Pharm Sci. 2017 Sep;30(5):1753-1757.
30. Kowalska-Krochmal B, Dudek-Wicher R. The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. Pathogens. 2021;10(2):165.
31. Kassa T, Al-Sayidi R H. Impact of antibiotics on public health. World J Exp Biosci. 2023;11:25-30.
32. Saleh FM, Saleh GM. Biofilm formation and antibiotic susceptibility for clinical and environmental isolates of *Pseudomonas aeruginosa*. World J Exp Biosci. 2015;3(1): 1-5.
33. Hamed SE, Al Shahwany AW. Increasing antimicrobial activity of some plant extracts against antibiotic resistant *Staphylococcus aureus* by using silver nanoparticles. World J Exp Biosci. 2016;4(1): 7-14.
34. Fontana R, Canepari P, Lleò MM, Satta G. Mechanisms of resistance of enterococci to beta-lactam antibiotics. Eur J Clin Microbiol Infect Dis. 1990 Feb;9(2):103-5. doi: 10.1007/BF01963633.
35. Sethuvel DPM, Bakthavatchalam YD, Karthik M, Irulappan M, Shrivastava R, Periasamy H, et al. β-Lactam resistance in ESKAPE pathogens mediated through modifications in penicillin-binding proteins: an overview. Infect Dis Ther. 2023;12(3), 829-41.
36. Liu F, Jin P, Gong H, Sun Z, Du L, Wang D. Antibacterial and antibiofilm activities of thyme oil against foodborne multiple antibiotics-resistant *Enterococcus faecalis*. Poult Sci. 2020;99(10):5127-36.
37. Bernardi S, Anderson A, Macchiarelli G, Hellwig E, Cieplik F, et al. Subinhibitory antibiotic concentrations enhance biofilm formation of clinical *Enterococcus faecalis* isolates. Antibiotics (Basel). 2021;10(7):874.
38. Pishkar SE, Nejat SH, Zarei-Yazdeli M, Estarki NSK. CA Review of genes related to biofilm formation in *Enterococcus*. Int. J. Enteric Pathog. 2024;12(1), 38-46.
39. Sillanpää J, Chang C, Singh KV, Montealegre MC, Nallapareddy SR, et al. Contribution of individual Ebp Pilus subunits of *Enterococcus faecalis* OG1RF to pilus biogenesis, biofilm formation and urinary tract infection. PLoS One. 2013;8(7):e68813.

40. Zgair AK, Chhibber S. *Stenotrophomonas maltophilia* flagellin is involved in bacterial adhesion and biofilm formation. *Microbiology*. 2013; *82:* 647-51.
41. Zgair AK, Chhibber S. Immunoassay method to check the flagellin mediated binding of *Stenotrophomonas maltophilia* to polystyrene. Mikrobiologiia. 2011;80(1):136-8.
42. Zgair AK, Chhibber S. Adhering ability of *Stenotrophomonas maltophilia* is dependent on growth conditions. Mikrobiologiia. 2011;80(4):459-64.