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Sub-inhibitory Concentrations of Nitrofurantoin Modulate *Enterococcus faecalis* Biofilm Formation and Adherence *in vitro*

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Abstract

Biofilm formation interferes with *Enterococcus faecalis* infections. Modulating biofilm formation may aid in developing innovative treatment strategies for multidrug-resistant bacteria responsible for urinary tract infections (UTIs). This study aims to evaluate the effect of nitrofurantoin minimum inhibitory concentration (MIC) on *E. faecalis* biofilm formation and adherence. Midstream urine samples (78) were collected from patients with UTI. The biochemical tests and VITIK technology were used to identify the *E. faecalis* isolates. The MICs of nitrofurantoin were determined to evaluate the susceptibility of *E. faecalis* isolates. Biofilm levels to polystyrene and adherence to human oral mucosal epithelial cells (OMECs) *in vitro* were evaluated. The inhibition role of nitrofurantoin sub-MICs in biofilm formation and adherence was examined. The incidence of UTI infection with *E. faecalis* was 12.82%. The susceptibility of ten *E. faecalis* isolates to nitrofurantoin, and their biofilm formation varied. No significant relationship was observed between the susceptibility of the isolates to nitrofurantoin and their biofilm production (r: +0.37, P>0.05). Sub-MICs of nitrofurantoin (½ MIC and ¼ MIC) significantly decline bacterial biofilm formation and adherence to human OMECs ($P < 0.05$). Nitrofurantoin sub-MICs reduce biofilm production and *E. faecalis* adherence to biotic and abiotic surfaces. This can help modulate treatment strategies in the future.

Keywords: Adhesion, Biofilm, *Enterococcus faecalis*, MICs, Nitrofurantoin.

تركيزات دون المثبطة من النتروفورانتوين تعدل تكوين الأغشية الحيوية لبكتيريا *Enterococcus faecalis* والتصاقها خارج الجسم

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الخلاصة

يتداخل تكوين الأغشية الحيوية مع عدوى *Enterococcus faecalis* قد يساعد تعديل تكوين الأغشية الحيوية في تطوير استراتيجيات علاج مبتكرة للبكتيريا المقاومة للأدوية المتعددة المسؤولة عن التهابات المسالك البولية. تهدف هذه الدراسة إلى تقييم تأثير الحد الأدنى لتركيز تثبيط النيتروفورانتوين (MIC) على تكوين الأغشية الحيوية لـ *E. faecalis* والالتصاق بها. تم جمع عينات البول (78) من مرضى يعانون من التهاب المسالك البولية. تم استخدام الاختبارات الكيميائية الحيوية وتقنية VITIK لتحديد عزلات *E. faecalis*. تم تحديد الحد

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الأدنى لتركيز تثبيط النيتروفورانتوين لتقييم قابلية عزلات *E. faecalis* للإصابة. تم تقييم مستويات الأغشية الحيوية للبوليسترين والالتصاق بالخلايا الظهارية المخاطية الفموية البشرية (OMECs) في المختبر استخدام تقنية الزرع النسيجي. تم فحص الدور المثبط للتركيزات الفرعية لتثبيط النيتروفورانتوين في تكوين الأغشية الحيوية والالتصاق بها. بلغت نسبة الإصابة بعدوى المسالك البولية بـ .12.82% *E. faecalis* وتباينت حساسية عشر عزلات من *E. faecalis* للنيتروفورانتوين وتكوين الأغشية الحيوية لديها. ولم تُلاحظ أي علاقة مهمة بين حساسية العزلات للنيتروفورانتوين وإنتاج الأغشية الحيوية لديها (r: +0.37 ، .(p>0.05وتؤدي مستويات التركيز الأدنى المثبط للنيتروفورانتوين MIC ½) ، و (MIC ¼إلى انخفاض كبير في تكوين الأغشية الحيوية البكتيرية والالتصاق بخلايا بطانة الأوعية الدموية البشرية .(p < 0.05) كما تعمل مستويات التركيز الأدنى الأدنى المثبط للنيتروفورانتوين على تقليل إنتاج الأغشية الحيوية والالتصاق بخلايا بطانة الأوعية الدموية البشرية .(p < 0.05)ويمكن أن يساعد هذا في تعديل استراتيجيات العلاج في المستقبل.

1. Introduction

Enterococcus faecalis is a Gram-positive streptococcal bacterium that poses considerable concern in medical microbiology due to its classification as a multidrug-resistant pathogen linked to nosocomial infections. It constitutes a component of the normal gut microbiota, yet it can precipitate serious endocarditis, bacteremia, septicemias, urinary tract infections (UTIs), and wound infections [1,2]. The capacity to develop biofilm is a significant problem in treating the infectious illnesses attributed to this bacterium. It can attach to biotic and abiotic surfaces due to its capacity to build biofilm and circumvent host defences, particularly the epithelial cells [3]. Biofilm production augments bacterial survival in adverse settings, facilitates chronic infections, and shields microorganisms from antibiotic treatment and host immunological defenses [4].

Nitrofurantoin is primarily used to treat uncomplicated urinary tract infections (UTIs). It is well-absorption by gut cells and targeting the bacterial DNA [5]. Using antibiotics at sub-inhibitory concentrations [sub-minimum inhibitory concentration (sub-MIC)] influences bacterial behavior, including biofilm formation, without necessarily inhibiting growth. Previous studies have indicated that sub-MICs of antibiotics promote bacterial virulence by inducing the expression of biofilm-related genes or facilitating adherence to different surfaces [6]. Recent studies showed that the sub-MICs of antibiotics reduced the biofilm formation and the ability of various species of pathogenic bacteria to adhere to biotic surfaces [7-9]. The pathogenicity of *E. faecalis* is a critical factor in studies on biofilm formation and adherence to biotic surfaces, as these processes play a key role in elucidating the mechanisms underlying treatment failures in clinical settings. Biofilm development augments pathogenicity and elevates bacterial resistance to antibiotic therapy [4]. Multiple genes contribute to the biofilm production of *E. faecalis*, including the *esp* (Enterococcal Surface Protein) gene. *esp* gene encodes a surface-associated protein, which is crucial in the first phase of biofilm development [10].

Comprehending the impact of sub-MIC concentrations of nitrofurantoin on biofilm development is crucial for individuals overseeing public health institutions and programs, as it may yield insights into innovative treatment options and guide clinical practice. *E. faecalis* possesses unique attributes that allow it to adapt in the presence of antimicrobial agents, such as its capacity for horizontal gene transfer and pseudomonal biofilm architecture, which complicates treatment efforts [11].

Previous studies have primarily focused on the bactericidal effects of nitrofurantoin against *E. faecalis* [12]. However, no prior study has investigated the modulation effect of low concentrations of nitrofurantoin on biofilm formation on abiotic and biotic surfaces, particularly in human epithelial cells. In the current study, we try to fill the gap in the knowledge regarding

the effect of non-inhibitory doses of nitrofurantoin on *E. faecalis* adherence to biotic and abiotic surfaces, and we also try to propose the mechanism of these effects.

2. Materials and methods

2.1. Bacterial isolates

The urine specimens were taken from patients (78 samples) with UTI (Al Hashimiya General Hospital, Babylon, Iraq). The patients were not treated with antibiotics 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) used ID-GPC card of the VITEK 2 to confirm the identification of the isolates. The *E. faecalis* isolates were maintained short-term by weekly sub-culturing onto nutrient agar [13,14]. The isolates were stored for a long term at -20 in a nutrient broth containing 20% glycerol [15].

2.2. Minimum inhibitory concentration (MIC)

The micro-dilution technique was used to measure the MICs of nitrofurantoin against 10 isolates of *E. faecalis* (Ef1, Ef2, Ef3, Ef4, Ef5, Ef7, Ef8, Ef9, Ef10, and Ef18). A hundred microliters of Mueller–Hinton broth (MHB; HiMedia, India) were added to each well of the U-bottom microtiter plate (96 wells microtiter plate). Two hundred micrograms of nitrofurantoin (Hikma Pharmaceuticals) were dissolved in 100 ml sterile MHB (HiMedia, India) to prepare a stock concentration (2 mg/ml). Double-fold serial dilutions (hundred microliters) were prepared in a U-bottom microtiter plate (96 wells microtiter plate) using sterile MHB starting from well No.1 to well No.12 of each row. Five microliters of standard inoculum of *E. faecalis* were added to the wells. The standard inoculum of *E. faecalis* was prepared by washing (thrice) the overnight growth (grown at 37 ºC in MHB) of bacteria with sterile phosphate-buffer saline (PBS; 0.1 M, pH 7.2) and centrifugation at 6,000 g for 12 minutes(Beckman Coulter, Brea, USA). The OD^{600nm} (optical density) of bacterial suspension was made to be 0.1 (Bioevopeak, Jinan, China). Plates were shaken gently and incubated at 37 ºC for 18 h. MHB and *E. faecalis* isolates (1^{st} control), the wells of MHB only (2^{nd} control), and different double dilutions of antibiotics only (3^{rd} control) were used in this experiment. The lowest antibiotic concentrations completely inhibit growth considered as MIC [9].

2.3. Biofilm formation

The previous method of Talib and Ghafil, was used to measure the biofilm formation for ten isolates of *E. faecalis*. In this experiment, 200 µl of sterile Tryptic Soy Broth (TSB, HiMedia) were put into flat-bottom (polystyrene) microtiter plate wells (96 sterile microtiter plates). The standard inoculum (5 µl) of *E. faecalis* was added to the wells and incubated at 37 ºC for 18 h. Standard inoculum was prepared by washing (thrice) the overnight growth (grown at 37 ºC in TSB) of bacteria with sterile PBS (0.1 M, pH 7.2) using centrifugation at 6,000 g for 12 min (Beckman Coulter, Brea, USA), the $OD^{600\ nm}$ was adjusted to 0.1 (Bioevopeak, Jinan, China). TSB was discarded, and the plates were washed thrice with sterile distilled water (D.W). The plates were dried (for 30 min at 55 ºC). Two hundred microliters of crystal violet (0.4%) were added to the wells (15 min at room temperature). The plates were re-washed five times with D.W. After drying the wells, 200 µl of anhydrous ethanol was added. The absorbency was measured at 570 nm (BioTek, USA). The experiment was repeated in triplicate [9].

2.4. Sub-MICs of nitrofurantoin and Biofilm

In this experiment, the effect of different concentrations of nitrofurantoin on the biofilm production of ten clinical isolates of *E. faecalis* was evaluated. The procedure of measuring biofilm formation was used with modifications. Instead of TSB, double-fold serial dilutions of sub-MICs of nitrofurantoin (½ MIC, ¼MIC, $^1/_8$MIC, $^1/_{16}$MIC, $^1/_{32}$MIC, and $^1/_{64}$ MIC) were prepared in TSB (Himedia) in the wells of a polystyrene microtiter plate (96 flat-bottom shape) were used. Five microliters of standard inoculum of *E. faecalis* (the method mentioned in the biofilm formation method) were added to the wells. The plates were incubated for 18 h at 37 ºC and then washed thrice with sterile distilled water. After completely drying at 55 ºC, the wells were stained with 200 µl of crystal violet (for 15 min). Then, they washed thrice with D.W. and dried again. Two hundred microliters of anhydrous ethanol were added to the wells. The optical density was measured at 570 nm (microplate spectrophotometer, BioTek, USA). The experiment was repeated three times [9].

2.5. Nitrofurantoin sub-MICs affect bacterial adherence to human OMECs (tissue culture)

The isolate of *E. faecalis* (Ef9), which produced the highest level of biofilm, was used to evaluate the effect of nitrofurantoin sub-MICs (up to $^1/_{64}$ MIC) on *E. faecalis* adherence to human OMECs. The previous method was followed to prepare human OMECs [9]. The bacteria colonies were grown onto TSB (Himedia). The $OD^{600\ nm}$ of *E. faecalis* was adjusted to 0.1 in TSB (the details mentioned in the biofilm formation method). The bacterial isolates were exposed to different nitrofurantoin sub-MICs (½ MIC, ¼ MIC, $^1/_8$ MIC, $^1/_{16}$ MIC, $^1/_{32}$ MIC, and $^1/_{64}$ MIC) for 24 h at 37°C and washed thrice with PBS (centrifugation at 6000 g for 12 minutes) and re-suspended in TSB. The optical density of treated bacteria was adjusted to 0.1 at 600nm. In tissue culture tubes (2ml tube, BIOFIL^R, China), 800 µl of 1×10^5 human OMECs suspended in Dulbecco's modified Eagle's medium [(D-MEM) containing 10% fetal calf serum, 10 mM L-glutamine] and 200 µl of *E. faecalis* that treated with nitrofurantoin were mixed and incubated for 2 h at 37 ºC. The human OMECs were washed thrice with PBS (0.1 M, pH, 7.2) using centrifugation at 1,000 g for 10 min (Beckman Coulter, Brea, USA). The human OMECs were lysed with PBS-0.5% Triton×100 (LOBA CHEMIE PVT. LTD). The plate count method was used to count the viable adhered bacteria. The untreated bacteria was used as a control [9].

2.6. Statistical analyses

The data was analyzed using Origin v. 8 software (OriginLab, Northampton, USA). It was shown as mean means ± standard deviation (SD). Student t-test was applied to evaluate group differences. Pearson's correlation coefficient (r) was used to check the relationship. P value<0.05 was reported as statistically significant [16].

3. Results

3.1. Bacterial Isolates

E. faecalis (10 isolates) were obtained from 78 urine specimens collected aseptically from UTI patients. 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 UTI with *E. faecalis* (12.82%).

3.2. Antibiotic Susceptibility

The MICs were measured for all isolates of *E. faecalis* (E f1, E f2, E f3, E f4, E f5, E f7, E f8, E f9, Ef 10, and E f18). The present study showed that the peak MIC of nitrofurantoin was against Ef18 (250 µg/ml) followed by Ef9 (125 µg/ml), while the lowermost MIC of nitrofurantoin was against Ef2, Ef4, Ef5, and Ef10 (15.6 µg/ml) (Figure 1).

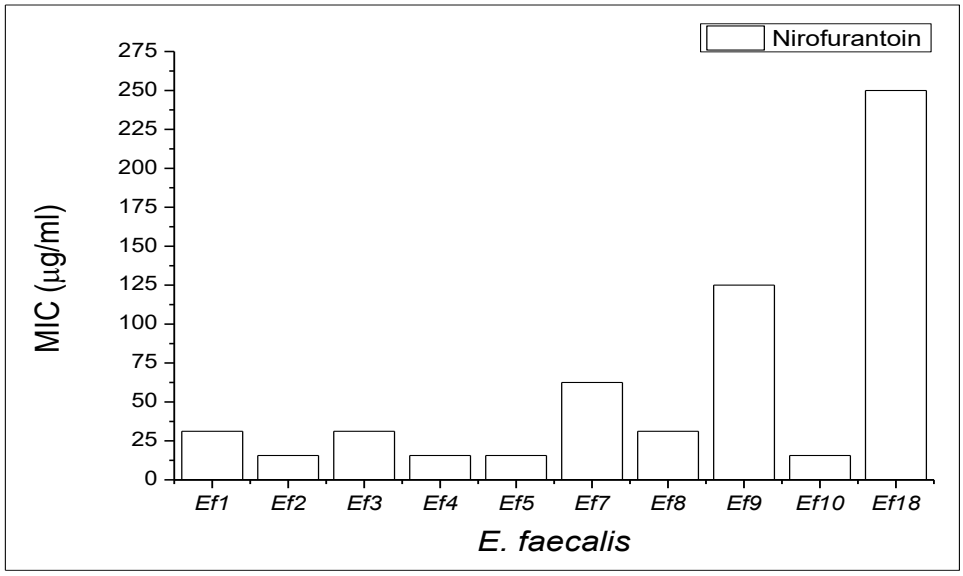

Figure 1: The MICs of nitrofurantoin against ten isolates of *E. faecalis* (Ef 1, Ef2, Ef3, Fe4, Ef5, Ef7, Ef8, Ef9, EF10, and Ef18).

3.3. Biofilm Formation and MICs

Figure 2 displays the levels of biofilm of 10 *E. faecalis* isolates. The *E. faecalis* (Ef 9) produced the highest level of biofilm (0.33 ± 0.07), followed by Ef 4 (0.23 ± 0.089). The lowermost biofilm was produced by Ef 8 (0.06 ± 0.02). The current study showed that all isolates produced the biofilm onto a polystyrene microtiter plate.

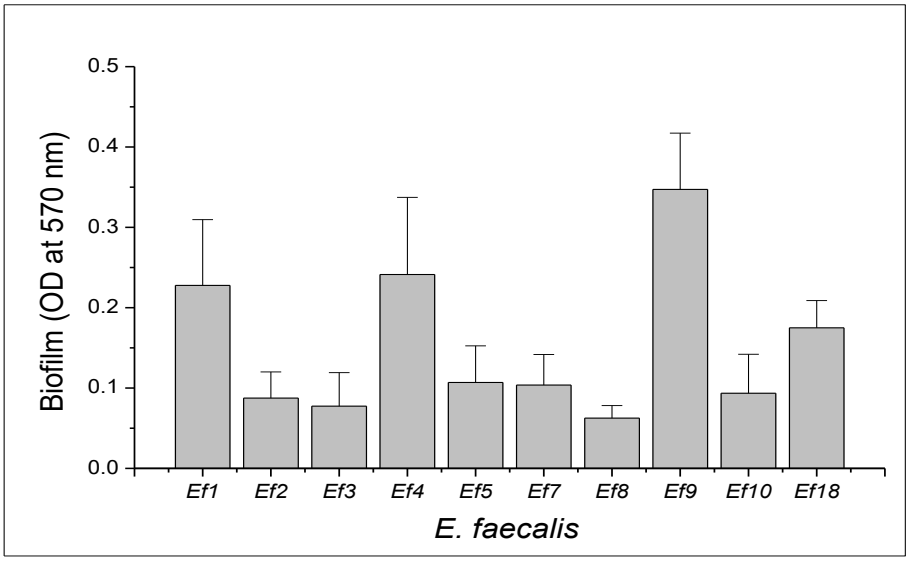

Figure 2: Biofilm production of ten *E. faecalis* isolates. A variety of biofilm levels has appeared. OD, optical density.

The relationship between the MICs of nitrofurantoin against ten isolates of *E. faecalis,* was involved in the current study. The study showed a positive correlation between the MIC of nitrofurantoin against ten *E. faecalis* isolates and the biofilm levels of the same isolates, but this correlation was not significant (r: +0.37, P>0.05) (Figure 3).

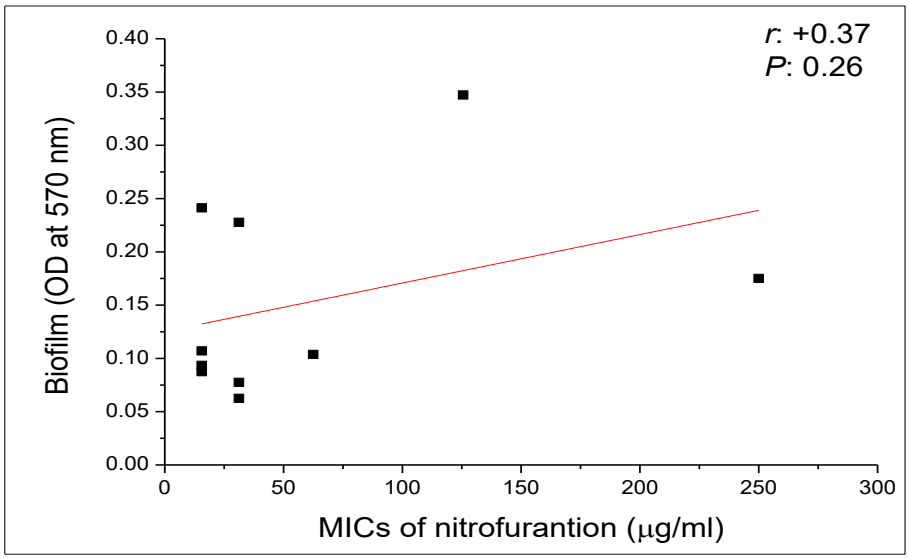

Figure 3: The relationship between biofilm formation of the ten isolates of *E. faecalis* and MICs of nitrofurantoin against the same isolates. r: Pearson correlation coefficient; P<0.05 considered a significant relationship.

3.4. Effect of nitrofurantoin Sub-MICs on Biofilm Formation

Sub-inhibitory concentrations effects of nitrofurantoin on the biofilm production of ten isolates of *E. faecalis* on polystyrene microtiter plates were evaluated. The results were expressed as the mean of the biofilm levels of the isolates of *E. faecalis* (Ef 1, Ef 2, Ef 3, Ef 4, Ef 5, Ef 7, Ef 8, Ef 9, Ef 10, and Ef 18) post-exposure to different concentrations of nitrofurantoin sub-MIC. The study demonstrated that nitrofurantoin sub-MICs (½ MIC and ¼ MIC) significantly reduced biofilm formation ($P < 0.05$) compared to the control (biofilm production of *E. faecalis* without exposure to nitrofurantoin). The treatment of the isolates to other nitrofurantoin sub-MICs ($^1/_8$ MIC, $^1/_{16}$ MIC, $^1/_{32}$ MIC, and $^1/_{64}$ MIC) did not significantly reduce (P>0.05) the biofilm level of the isolates of *E. faecalis* on polystyrene (Figure 4).

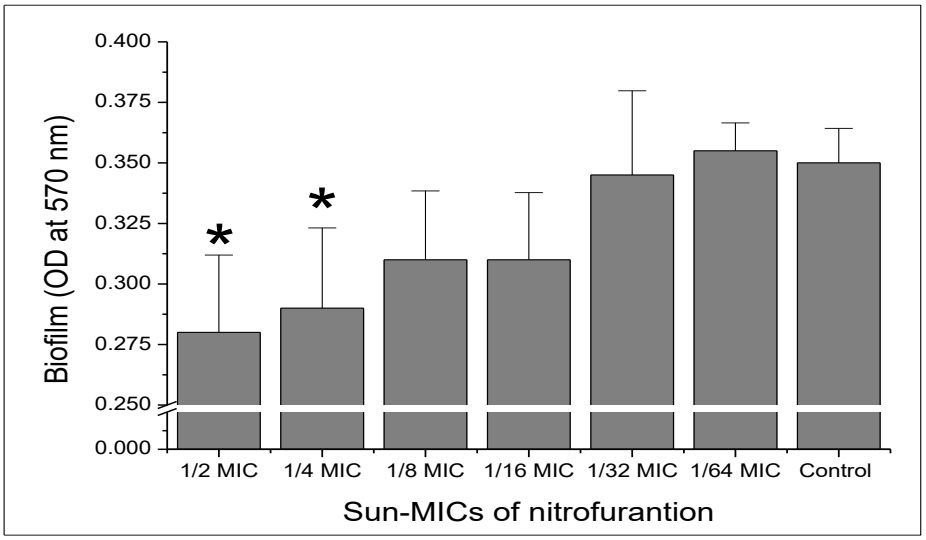

Figure 4: Biofilm formation of ten isolates of *E. faecalis* post-exposing to different concentrations of nitrofurantoin sub-MICs (from ½ MIC to $^1/_{64}$ MIC). Results were expressed in the average biofilm formation of ten isolates and standard deviation [mean ± SD (standard deviation)]. Asterisks indicate a significant difference from the control (biofilm level *according to optical density (OD) at* 570 nm without nitrofurantoin stress).

3.5. Nitrofurantoin sub-MICs modulate E. faecalis adherence to human OMECs

In the present study, the human OMECs (biotic model) were used to evaluate the impact of different sub-MICs of nitrofurantoin (½ MIC, ¼ MIC, $^1/_8$ MIC, $^1/_{16}$ MIC, $^1/_{32}$ MIC, and $^1/_{64}$ MIC) on Ef 9 (the *E. faecalis* that produced the highest level of the biofilm formation) adherence to biotic surfaces. The viable bacterial count was used to estimate the count of live bacteria that adhered to the human OMECs (Figure 5). The results were compared with the viable bacterial count of Ef 9 that adhered to human OMECs without antibiotic stress. The study showed the highest significant decline in bacteria adherence was seen when the bacteria were pretreated with ½ MIC and ¼ MIC of nitrofurantoin ($P<0.05$). The other sub-MICs of nitrofurantoin ($^1/_8$ MIC, $^1/_{16}$ MIC, $^1/_{32}$ MIC, and $^1/_{64}$ MIC) did not decrease bacterial adherence significantly ($P>0.05$).

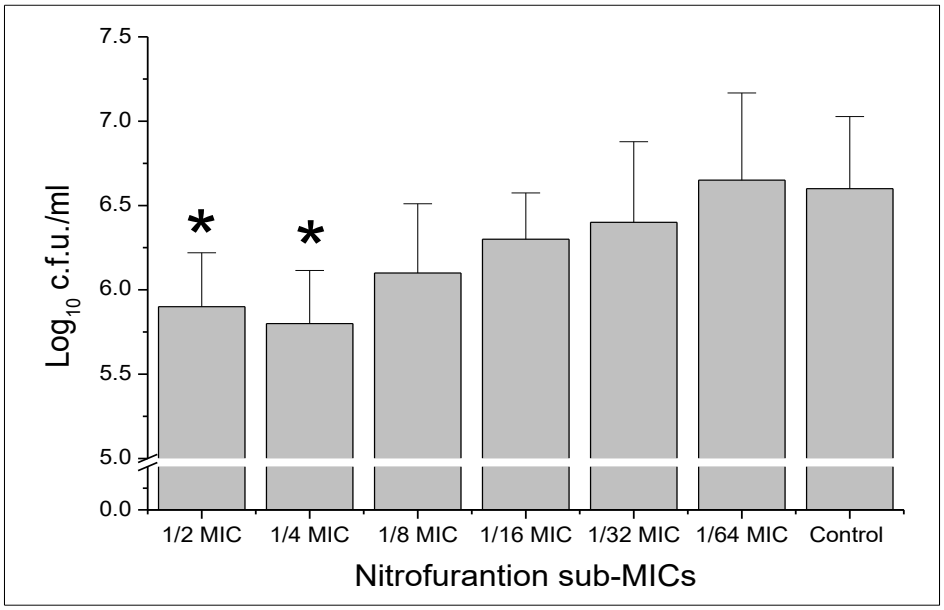

Figure 5: The viable bacterial count of *E. faecalis* (Ef 9) adhered to human OMECs post-exposure to different sub-MICs of nitrofurantoin (from ½ MIC to $^1/_{64}$ MIC). The control is the viable count of *E. faecalis* (Ef9) adhered to human OMECs post-exposure to PBS). *, a significant difference from the adherence to control. $P<0.05$ is considered a significant difference.

3. Discussion

Biomass production is a significant factor contributing to their persistence and virulence, particularly in nosocomial infections that exhibit bacteria's resistance to a wide spectrum of antibiotics. Earlier studies have shown that sub-inhibitory concentrations of antibiotics variably affect bacterial biofilm formation [7-9,17,18]. Investigating the role of sub-MICs of nitrofurantoin in modulating *E. faecalis* biofilms may provide appreciated insights into developing more effective treatment strategies and the impact of low antibiotic concentrations on treating *E. faecalis* infectious diseases [19].

The study investigated the isolation of ten *E. faecalis* isolates from urine specimens of patients suffering from UTIs. The incidence of infection with these bacterial isolates was relatively high. The susceptibility of the isolates to nitrofurantoin in terms of the MIC method was assessed, revealing variable responses among the isolates to this antibiotic. Similarly, the biofilm-forming ability of the isolates also exhibited variability. No significant relationship was seen between the biofilm levels of the isolates and their susceptibility to nitrofurantoin. The study further demonstrated that exposure of these isolates to sub-MICs of nitrofurantoin

reduced the biofilm production to the abiotic surface and adherence to biotic surfaces (human OMECs), particularly at higher sub-MIC levels.

The studies that deal with the effect of sub-MICs of nitrofurantoin on the *E. faecalis* biofilm production *in vitro* are scanty in the literature. Caixeta Magalhães Tibúrcio *et al.,* reported that 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 level of *E. faecalis* [17]. Bernardi *et al.,* investigated the effect of sub-inhibitory concentrations of different antibiotics on the level of biofilm produced by *E. faecalis*; they found that the sub-MICs of the studied antibiotics enhanced the biofilm formation *in vitro* [19]. In contrast, previous studies reported that the sub-MICs of different antibiotics reduce the biofilm formation of clinical pathogenic bacteria *in vitro* [7-9].

The reduction in biofilm production and adherence of bacteria observed in this study may be attributed to the interference of nitrofurantoin with key mechanisms involved in biofilm formation, such as quorum sensing (QS), gelatinase (GelE), cytolysin, enterococcal surface protein (Esp), and the role of pili and flagella [17,20-24]. The findings highlight that sub-MIC levels of nitrofurantoin interfere with biofilm formation, suggesting their potential as adjunct therapies to combat biofilm-associated infections and improve treatment outcomes in infected burn patients. Further research is necessary to elucidate 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 [25,26].

This study also opens avenues for future investigations into the effects of low nitrofurantoin concentrations on bacterial virulence, particularly their biofilm-forming ability, under both *in vitro* and *in vivo* conditions. Additionally, exploring the influence of these antibiotics at different stages of biofilm formation could provide a deeper understanding of their therapeutic potential.

4. Conclusion

The incidence of UTI by *E. faecalis* is documented here. The susceptibility of clinical isolates of *E. faecalis* to nitrofurantoin is variable according to the isolates. All studied isolates of *E. faecalis* were capable of forming biofilms on polystyrene microtiter plates; however, their biofilm-forming ability was not correlated with their susceptibility to nitrofurantoin. The impact of nitrofurantoin sub-MICs on biofilm production was a concentration-dependent manner. The ½ and ¼ MICs reduced the biofilm formation significantly. However, the lowest concentrations did not significantly reduce biofilm formation to polystyrene. A similar finding was seen when the impact of different nitrofurantoin sub-MICs on adherence of *E. faecalis* to human OMECs *in vitro*.

Ethical 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/1124/0098 on June. 8, 2024.

Conflict of interest

The authors declare that there is no conflict of interest related to the current project.

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