Investigation of gelatinase gene expression and growth of E. faecalis clinical isolates in biofilm models

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INTRODUCTION: Enterococcus faecalis is major reason of biofilm related infections and also interact with Staphylococcus aureus in biofilms. Gelatinase (gelE) enzyme is an important virulence factor of E. faecalis for biofilm formation. This study aimed to compare the biofilm producing E. faecalis isolates from urine and urinary catheters. Influence of S. aureus on the growth of E. faecalis biofilm cells was also investigated in a dual biofilm model in vitro. Another aim was to evaluate E. faecalis gelE gene expression during biofilm formation.

METHODS: Firstly, crystal violet staining was used to measure the total biofilm biomass of the isolates. Secondly, plate counting method was performed to determine the biofilm formation ability of E. faecalis isolates and the effect of S. aureus on E. faecalis biofilm formation. Finally, GelE expression profile of the isolates was assessed by qRT-PCR.

RESULTS: According to crystal violet staining and plate counting method, all E. faecalis isolates were found to be biofilm producers and the number of E. faecalis sessile cells were increased in the presence of S. aureus. Among 21 of E. faecalis isolates, ten of them expressed high levels of gelE gene, however eight of them had low expression profile (P < 0.05).

DISCUSSION AND CONCLUSION: When they grow up together, S. aureus may give some advantages to E. faecalis as increasing the sessile cell growth. The expression of gelE gene has not been affected by E. faecalis biofilm formation of the isolates collected from the patients with urinary tract infections.

Keywords: Dual biofilm, E. faecalis, S. aureus, gelatinase, qRT-PCR.
1. Introduction

Biofilms are defined as biotic or abiotic surface-attached microbial consortium and have multiple stages as initial reversible attachment, production of extracellular polymeric matrix (EPM) including proteins, polysaccharides, nucleic acids, irreversible attachment, etc.\textsuperscript{1,2} Biofilm formation is an important problem causing failure in antimicrobial treatment because sessile cells in biofilm are highly resistant against antimicrobial agents. It has been highlighted that 65 – 80% of all infections are biofilm-related. Biofilm cells are phenotypically, physiologically and genotypically different from non-attached (planktonic) cells. Moreover, high concentrations of antimicrobial agents are necessary to kill sessile cells in a mature biofilm vs. planktonic cells.\textsuperscript{3}

It has been recently shown that, most of diseases are occurred by polymicrobial communities.\textsuperscript{4-8} Although some infections considered as predominantly monomicrobial, they may be influenced by the other microorganismal association during active infection.\textsuperscript{4} The physiology of microbial cells in the biofilm has been frequently changed by these interactions and leads to obtain various advantages, such as resistance to antimicrobials or human immune system, metabolic cooperation, quorum sensing systems, more productive gene sharing.\textsuperscript{9-12}

Enterococci species have been recognized as opportunistic pathogens for many nosocomial infections and are natural inhabitants of the human intestinal and oral flora. Enterococcus faecalis is the most common
species leading to many infections among the other enterococcus species. They can readily form biofilms and keep growing on various medical devices surfaces such as urinary catheters despite a serious inflammatory response. S. aureus has become an important problem of hospital acquired infection associated with indwelling medical devices and surgical wounds. It may cause chronic infections not to be treated with antibiotics because of the ineffective host immune response. Moreover, staphylococci have non-specific resistance mechanisms such as biofilm formation.

The changing expression levels of virulence factors of E. faecalis has been shown even they formed biofilm or not. Among the virulence factors, the gelatinase enzyme is one of the important factor that hydrolyzes gelatin, casein and collagen. Although there have been many studies on biofilm formation and gelE expression by E. faecalis, it has been still not clear how the gelE expression levels changes in mono or polymicrobial biofilms.

In this study, we evaluated the biofilm ability of the E. faecalis isolates by quantification assays and then we set up an in vitro dual biofilm model in a repeatable style and determined the influence of the presence of S. aureus on the growth of E. faecalis by plating assay. Finally, the gelE gene expression levels of E. faecalis was measured by qRT–PCR.
2. Materials and Methods

2.1. Strains used in the study

Totally, 20 *E. faecalis* clinical isolates and a strain as positive control (*E. faecalis* ATCC 29212) were used in this study. These isolates were taken from urinary catheter (n = 10) and urine samples (n = 10) of the hospitalized intensive care unit patients who are admitted to a University Hospital from 2000 to 2011. For dual biofilm formation; all the *E. faecalis* isolates and *E. faecalis* ATCC 29212 were cultured with *Staphylococcus aureus* ATCC 29213.

2.2. Mono and dual biofilm formation in microtiter plates

Final inoculum suspensions of all clinical *E. faecalis* strains were adjusted to approximately $10^6$ colony forming unit (CFU) ml$^{-1}$. Each experiment included the biofilm-forming *E. faecalis* ATCC 29212 strain as a positive control. For dual species biofilms, *E. faecalis* isolates were co-cultured with a laboratory strain of *S. aureus* ($10^6$ cfu / ml) and incubated at 37°C without shaking. Sterile triptic soy broth (TSB) (Becton Dickinson GmbH, Heidelberg, / Germany) with 0.25% glucose was used as a blank. For each test condition, 12 wells of a flat-bottomed polystyrene 96-well microtiter plate were inoculated with 100 μl of the final inoculum suspension. After four hours of incubation at 37°C without shaking, non-adhered cells were removed and rinsed with 100 μl of 0.9% physiological saline (PS), then 100 μl of fresh TSB with 0.25% glucose was added and the plates were incubated for an additional 20 hours.
for biofilm maturation. After 24 hours, supernatants were removed and each well was rinsed with PS before quantifying the sessile cells.

2.3. Quantification of the biofilms

2.3.1. Crystal Violet staining

The biomass quantification of *E. faecalis* biofilms was performed according to an optimized assay. After washing with sterile PBS, the wells were stained with 100 μl of a solution of 0.2% crystal violet for 15 min. The stained biofilms were rinsed again three times with PBS to remove excess dye and dried for 15 min. at room temperature. The bound dye was solubilized in 150 μl of acetone/ethanol solution. The optical densities (ODs) of the stained adherent cells were read at 570 nm using a micro-ELISA plate reader. We defined the cut-off OD (0.282) as three standard deviations above mean OD of the negative control. Each isolate was tested in twelve wells in each assay and each assay was carried out in duplicate (n = 24).

2.3.2. Plate counting

The quantification of the number of cells in mature biofilms was done via plate counting using triptic soy agar (TSA) medium. Biofilms were detached by vortexing (five min.) followed by sonication (five min.). The sonicated fluids were serially diluted and plated on TSA to determine the number of CFU per ml of the isolates. Bile esculin azide agar was used for plating of *E. faecalis* isolates in mature dual biofilms that were formed by *E. faecalis* and *S. aureus*.

2.4. Expression of the gelE gene in planktonic and biofilm cells of *E. faecalis*
Total RNA was extracted from the mono and dual-species biofilm cells with the RNeasy® Mini Kit according to the manufacturer’s recommendations (Qiagen GmbH, Germany). All RNA extracts were prepared as 100 ng μl⁻¹ per sample and transcribed into cDNA using Transcriptor High Fidelity cDNA Synthesis Kit according to the manufacturer’s instructions (Roche Diagnostics GmbH, Germany). Real-time PCR (Roche Light Cycler 2.0) was performed with LightCycler Faststart DNA Master SYBR Green1 (Roche Diagnostics GmbH) in a total volume of 20 μl. Primer sequences for the housekeeping gene 16sRNA and gelE were obtained from the literature and listed in Table (Otto, 2008). The 16sRNA gene was used to normalize the expression level of gelE. Melt curve analysis was carried out to assess the specificity of each primer pair. The comparative CT method for relative quantification (ΔΔCT method) was performed to analyze the data.

2.5. Statistical analysis

The independent samples t test was used to compare biofilm cell CFU counts between 2 groups (with / without S. aureus biofilms). One-way analysis of variance (ANOVA) was used to evaluate CFU differences within a group. The CFU counts were log-transformed before the statistical tests. P value < 0.05 was considered significant. For gene expression, the results were analyzed by t-tests and only differences of more than two fold up- or down- regulation and with a P-value < 0.05 were considered significant.
Ethics Committee Approval

The authors declared that there is no need an ethics committee approval for this study.

3. Results

3.1. Detection of biofilm production by *E. faecalis* isolates

Totally 20 *E. faecalis* isolates were analyzed to determine the ability of biofilm formation. All of the isolates were found to be biofilm-positive by plate counting and crystal violet staining (Figure 1 and 2). In terms of biofilm forming ability no statistically significant difference was determined between the isolates from the catheters and without catheters (Figure 2).

In the co-culture of *E. facealis* with *S. aureus*, the cell counts of *E. facialis* were significantly higher than in their mono-species biofilm (Figure 2). Our results showed that *S. aureus* contributed the growth of *E. faecalis* biofilm cells with unknown mechanism.

3.2. GelE gene expression in planktonic and biofilm cells of *E. faecalis* clinical isolates

We used qRT-PCR to compare the expression levels of gelE in planktonic and biofilm cells of 21 *E. faecalis* isolates (including positive control) from the urine and urinary catheter samples of hospitalized patients and *E. faecalis* ATCC 29212. According to the results obtained from the mRNA levels of gelatinase in planktonic and biofilm cell of *E. faecalis*, twelve of twenty-one *E. faecalis* strains (including positive control) exhibited increased gelE gene expression, however,
only ten of them (including positive control) were statistically significant (P < 0.05). Eight isolates showed significantly decreased expression levels (P < 0.05) (Figure 3).

4. Discussion

Biofilm related urinary tract infections represent the main cause of nosocomial infections. Enterococci (especially *E. faecalis*) and *Staphylococcus aureus* are one of the major challenging problems for treatment of urinary tract infection.24 It is widely known that the presence of bacterial biofilms on the inner or outer surface of the catheter leads to catheter-associated urinary tract infections (CAUTIs).15 The occurrence of CAUTIs, as the most common hospital acquired infection, has an important economic and clinical impact and is directly related to the majority of uropathogens such as *E. faecalis* and *S. aureus* that may form biofilms. In the current study, we assessed the ability of biofilm formation of clinical *E. faecalis* isolates in alone and co-culture with *S. aureus* in vitro. Our results indicated that all isolates from inpatients with and without urinary catheters were biofilm positive with regard to the plate counting and crystal violet staining methods (Figure 1 and 2). The starting bacteria concentration was normalized as 6 log$_{10}$ (10$^6$ cfu / ml). However, after the incubation period, the lowest bacteria number in the well plates were found to be 8.7 log$_{10}$. This results showed that all the mono-species biofilm isolates of *E. faecalis* attached and grew on the walls of the wells in microtiter plates (Figure 2).
Interspecies interactions in polyspecies biofilm usually provide various advantages for the inhabitant species such as increased tolerance against several antimicrobials, increased virulence in infections. Pastar et al. showed that the presence of *Pseudomonas* inhibited the growth of *S. aureus* in vitro and induced expression of *S. aureus* virulence factors in polymicrobial wound infection. In another study, the effect of *Streptococcus mutans* on *E. faecalis* biofilm formation was investigated and increase of biofilm formation of *E. faecalis* by *S. mutans* was obtained. It has been previously shown that the combined effect of *C. albicans* and *E. faecalis* in a mice model resulted in increasing the growth of enterococci in animals when *C. albicans* had been introduced. In a *P. aeruginosa* and *C. albicans* dual biofilm model, it has been observed that *P. aeruginosa* formed biofilms on the fungal filaments of *C. albicans* and this close contact caused the killing of the fungal filaments. In our study, the number of sessile cells of *E. facealis* in dual-species biofilms with *S. aureus*, displayed significantly higher than in their mono-species biofilm. We concluded that the growth and biofilm formation of the *E. faecalis* isolates were increased by *S. aureus* sessile cells. According to the biofilm cell counts between urine and urinary catheter samples, the counts of *E. faecalis* isolates from urinary catheters was found more than the isolates from urine.

Many virulence factors have significant roles in the pathogenesis of Enterococcal infections such as adhesion, colonization, invasion. Although it has been
indicated that some of the major virulence genes were related with biofilm formation on abiotic surfaces in hospital environment, some research on virulence mechanism and related genes in biofilm formation have been still needed. The high amount of gelE gene expression in *E. faecalis* biofilm cells was shown in some studies, whereas others were in contradiction with this finding. Arciola et al. showed importance of gelatinase in biofilm formation in implant infections. In a recent study, the prevalence of the gelE gene was determined as 64.3% among 510 clinical Enterococcus spp. isolates from UTI and wound infections. However, Kafil et al. did not find the significant effect of the presence or absence of gelatinase on the biofilm production by Enterococci species. We examined the *gelE* mRNA levels of both planktonic and sessile cells of *E. faecalis* ATCC 29212 and 20 *E. faecalis* isolates by RT-qPCR. Our results showed that the *gelE* expression levels of ten isolates were significantly enhanced, however eight of isolates were significantly decreased in biofilms when compared to their planktonic forms (P ≤ 0.05) (Figure 3). According to this results, we concluded that the *gelE* expression had not an effect on biofilm formation of the isolates collected from urinary tract infections (P > 0.05) (Figure 3). The comparison of *gelE* mRNA levels of the isolates from two different samples showed no significant difference as well.

5. Conclusions:
There was no statistically significance between the isolates from the catheters and without catheters in
terms of biofilm forming capability. *E. faecalis* sessile cells counts were increased in the presence of *S. aureus*. The expression of gelE gene has not been affected by *E. faecalis* biofilm formation of the isolates collected from the patients with urinary tract infections.

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**Conflicts of interest**
The authors declare that they have no conflicts of interest.

**Financial disclosure:**
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**References**


**Figure 1** Biofilm forming ability of the *E. faecalis* isolates from the urine and urinary catheter samples of hospitalized patients by crystal violet staining assay.

a Biofilm formation degrees of the isolates were determined by crystal violet staining assay. Results are means of at least three different experiments. OD: optical density.
Figure 2: Biofilm forming ability of the *E. faecalis* isolates in mono and dual biofilms

A)

![Graph A](image1)

B)

![Graph B](image2)

Numbers of the sessile cells of the *E. faecalis* isolates in mono and dual species biofilms were determined by plate counting assay. A: The results of isolates from the urinary catheter samples, B: The results of isolates from the urine samples. Results are means of at least three different experiments. Mono: only *E. faecalis* biofilms, Dual: *E. faecalis* and *S. aureus* biofilms, CFU: colony forming unit, *: statistically significant.
Figure 3. Gelatinase gene relative expression ratios of *Enterococcus faecalis* ATCC 29212 and clinical isolates in planktonic and biofilm cells.

A) B) Melting curves of gelE and 16srRNA (housekeeping gene) of the bacteria, respectively.

*: Statistically significant (P-value < 0.05)

**Table**: The primers for quantifying the genes of *E. faecalis* by RT–qPCR
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<tr>
<td>16sRNA</td>
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