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Genotype–phenotype correlation of fecal *Streptococcus* regulator (*fsr*) locus with gelatinase activity and biofilm formation intensity in clinical *E. faecalis* isolates

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Abstract

Background Enterococci, known for their disturbing involvement in nosocomial infections, possess a diverse set of virulence factors, regulated by multiple genes. A key virulence regulator is the fecal *Streptococcus* regulator (Fsr) quorum sensing system. Multiple reports describe the involvement of *fsr* genes in several virulence mechanisms, notably gelatinase production and biofilm formation; however, the presence of *fsr* genes does not necessarily predict those virulence phenotypes. This study investigates the factors affecting the relation between molecular detection of *fsr* genes and accurate prediction of gelatinase activity and biofilm formation intensity.

Methods One hundred enterococcal samples were collected from patients suffering from urinary tract infections. The isolates were identified through the use of a polymerase chain reaction (PCR) technique targeting the *ddl* gene. Biofilm formation was quantified by the crystal violet assay, while gelatinase activity was evaluated on gelatin agar plates. PCR was used to detect the *fsr*A and *fsr*B genes, as well as the gelatinase enzyme-encoding gene (*gel*E).

Results Out of the collected 100 isolates, 93% were identified as *Enterococcus faecalis*. The isolates formed biofilm with different intensities: 47% were strong biofilm producers, 28% moderate, and 21% weak, while only four isolates (4%) did not form biofilm. Only 14% of all isolates had detectable gelatinase activity. The *fsr*A and *fsr*B genes were detected in 26% and 28% of the tested isolates, respectively, while *gel*E was detected in 57% of the isolates. Whereas no association was found between biofilm formation intensity and *fsr* locus genes or gelatinase activity, a strong positive correlation ($r = 1$) was found between the detection of both *fsr*A and *fsr*B genes and the gelatinase activity.

Conclusion *fsr*A and *fsr*B have a diagnostic value and may be used as biomarkers for gelatinase activity in *E. faecalis*.

Keywords Quorum sensing, Gelatinase, Biofilm intensity, *Enterococcus faecalis*

Background

Enterococci are Gram-positive members of the intestinal microbiota; however, they frequently act as opportunistic pathogens with the ability to cause community- and hospital-acquired infections. *E. faecalis* and *E. faecium* are the enterococcal species most frequently linked to infections [1].

Infections of the urinary tract represent the most prevalent type of infection caused by *Enterococcus* spp., which is responsible for more than 30% of nosocomial urinary

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tract infections and has been identified as the second most common pathogen after *Escherichia coli* in catheter-associated urinary tract infections [2].

To be able to cause such diseases, enterococci possess several virulence factors that give them an advantage over their host's immune system. Quorum sensing is one of the important virulence mechanisms, and can be regulated by the *fsr* locus, made up of *fsr*A, *fsr*B, *fsr*D, and *fsr*C genes [3]. FsrA protein has a DNA-binding domain from the LytTR family [4]. The attachment of phosphorylated FsrA to LytTR-binding sites located in the regions preceding *fsr*B and *gel*E suggests that FsrA acts as the response regulator within this control system [4]. FsrB, a protein found within the cell membrane, is a member of the accessory gene regulator protein B family. It is responsible for transforming FsrD into an active form that stimulates the production of gelatinase, through the activation of the pheromone GBAP, which is subsequently exported outside of the cell [5]. The fourth gene within this locus, *fsr*C, is responsible for coding the transmembrane histidine kinase, FsrC, serving as the sensor-transmitter for the *fsr* operon [4].

The *fsr* locus regulates the synthesis of the gelatinase and serine protease enzymes, in addition to regulating the expression of EF1097 and EF1097b genes, which encode for enterocin [4]. Studies of the transcriptome have shown that, beyond its role in controlling *gel*E, *spr*E, and EF1097, the Fsr system plays a part in regulating approximately 75 other genes. These include genes associated with surface proteins (EbpR), biofilm formation (BopD), and various metabolic activities [6]. These proteins are engaged in a variety of biological processes and contribute to *E. faecalis* virulence and pathogenicity [3].

Downstream of the *fsr* locus lies the gene responsible for gelatinase production (*gel*E gene) [3]; The gelatinase enzyme, produced by *E. faecalis*, serves as a crucial virulence factor, enabling it to break down gelatin, collagen, casein, hemoglobin, and other peptides [7]. This enzyme facilitates the degradation of host tissues, aiding *E. faecalis* in colonizing and infiltrating host structures [8].

Quorum sensing also regulates another important virulence mechanism, which is biofilm formation. A biofilm is a community of microorganisms that are adhered to either living (biotic) or non-living (abiotic) surfaces, usually encased within a protective layer made up of extracellular polymeric substances [9]. Biofilm formation is an important element of *E. faecalis* pathogenicity because it helps the bacteria colonize a variety of settings, including host tissues and medical devices [10].

In addition to quorum sensing, gelatinase activity has also been linked to biofilm formation through degrading other bacterial cells and stimulating the release of AtlA, the major autolysin involved in biofilm formation [11].

A critical gap still remains in our understanding of the relationship between molecular detection of *fsr* genes and the accurate prediction of gelatinase activity, as well as biofilm formation intensity in enterococcal infections. The observed discrepancy between the presence of the *gel*E gene and actual gelatinase activity, along with the ambiguous role of the *fsr* locus in biofilm formation intensity, underscores the complexity of translating molecular diagnostics into effective clinical predictions and treatment strategies for *Enterococcus*-related infections.

Methods

Bacterial strains and culture condition

E. faecalis ATCC 29212 and *Bacillus subtilis* ATCC 6633 were used as positive control strains. One hundred isolates were retrospectively collected from Al-Borg Medical Laboratories from patients in Egypt diagnosed with urinary tract infections during the period of 2020 to 2021. These isolates were preserved at −80 °C in Brain Heart Infusion broth supplemented with 25% glycerol. For culturing purposes, samples from the stock were isolated on bile esculin agar and incubated at 37 °C overnight.

Identification of bacterial isolates

Pure bacterial colonies were obtained through surface streaking on bile esculin agar and identified to the genus level using Gram staining, catalase test, and their ability to tolerate 6.5% NaCl broth.

Identification of the enterococci to the species level

DNA extraction

DNA from the isolates under examination was extracted using the boiling method [12], wherein 3 to 5 well-separated colonies from the culture being tested were suspended in 100 µL of nuclease-free water and subjected to heating at 100 °C for 5 min. Following this, the suspension was rapidly cooled to −20 °C for 5 min and then centrifuged at 10,000 rpm for 10 min. The supernatant, which contained the crude DNA extract, was then stored at −20 °C for preservation.

Polymerase chain reaction (PCR)

The PCR was conducted in a total reaction volume of 50 µL, which included 25 µL of DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 10 pmol of both the forward and reverse primers specific to the *ddl* genes (Table 1), and 2.5 µL of the extracted crude DNA. The multiplex PCR process encompassed an initial denaturation phase of 2 min at 95 °C, followed by 30 cycles that each included a denaturation step at 95 °C for 30 s, an annealing step at 52 °C for 30 s, and an extension step at

Table 1 Primers used in this study and their sequence

Target gene	Primer pair	Amplicon size (bp)	References
ddl of *E. faecalis*	Forward: ATCAAGTACAGTTAGTCTTTA	942	[13]
	Reverse: AACGATTCAAAGCTAACT		
ddl of *E. faecium*	Forward: CCAAGGCTTCTTAGAGA	535	[13]
	Reverse: CATCGTGTAAGCTAACTTC		
*fsr*A	Forward: CGTTCCGTCTCTCATAGTTA	474	[14]
	Reverse: GCAGGATTTGAGGTTGCTAA		
*fsr*B	Forward: TAATCTAGGCTTAGTTCCCAC	428	[14]
	Reverse: CTAAATGGCTCTGTCGTCTAG		
*gel*E	Forward: GGTGAAGAAGTTACTCTGAC	704	[14]
	Reverse: GGTATTGAGTTATGAGGGGC		

72 °C for 30 s. The procedure was concluded with a final extension step that lasted for 5 min at 72 °C [13].

The PCR products were visualized by electrophoresis on a 1.5% agarose gel (w/v), stained with ethidium bromide, using a electrophoresis system (Mupid exU, Japan). A Generuler 100 bp DNA ladder (Fermentas, Germany) served as the marker. The presence of the *ddl* gene in *E. faecalis* and *E. faecium* was confirmed by the visualization of 942 bp and 535 bp bands, respectively. *E. faecalis* ATCC 29212 was used as the positive control in these experiments.

Biofilm assay

Biofilm formation was evaluated through the use of the crystal violet assay method [15], with certain modifications. The process for assessing biofilm formation began with inoculating an overnight culture of the tested isolates into trypticase soy broth (TSB) enriched with 1% glucose, followed by a 24-h incubation at 37 °C. Post-incubation, the optical density (OD) of the culture was adjusted to a 0.5 McFarland standard, with further dilution of the cultures to a 1:100 ratio in TSB. This diluted culture (200 µL) was then transferred to the wells of a sterile flat-bottomed 96-well plate and incubated again at 37 °C for 24 h.

After this incubation, the contents of the wells were discarded, and the wells were washed with saline and left to dry. The biofilm, comprised of adherent microbial cells, was fixed with absolute methanol and then stained with a 0.1% crystal violet solution for 15 min. Subsequent to the staining, the excess crystal violet was removed, and the wells were washed with distilled water before the plates were left to dry.

The adhered stain was dissolved using 33% glacial acetic acid, and the OD of the dissolved stain was measured at 570 nm using a plate reader (Unicam, UK). TSB containing 1% glucose without tested isolate served as the negative control. The experiment was conducted in triplicate.

The degree of biofilm formation by each isolate was classified based on the OD of the dissolved stain as follows: strong biofilm formation was indicated if $OD > 4 \times OD.c$, moderate biofilm formation if $2 \times OD.c < OD \leq 4 \times OD.c$, and weak biofilm formation if $OD.c < OD \leq 2 \times OD.c$, where OD.c represents the optical density of the negative control plus three times the standard deviation of the negative control [16].

Gelatinase assay

Gelatinase production was assessed by the nutrient gelatin plate method [17], with slight adjustments. In summary, the isolates under investigation were streaked onto the surface of nutrient agar plates that were supplemented with 5% gelatin. These plates were then incubated at 37°C for a 24-h period. Following the incubation, Frazier solution was applied dropwise onto the agar surface. The appearance of a clear zone surrounding the microbial growth served as an indicator of positive gelatinase activity. *Bacillus subtilis* ATCC 6633 served as the positive control for the experiment.

Genotypic screening for *fsr*A, *fsr*B, and *gel*E genes

For the detection of the *fsr*A, *fsr*B, and *gel*E genes, PCR was used. The PCR protocol for each reaction started with an initial denaturation phase at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s. Annealing temperatures were set at 50 °C for *fsr*A, 49 °C for *fsr*B, and 47 °C for *gel*E gene detection (Table 1), each for 30 s, with an extension at 72 °C for 30 s. This was concluded by a final extension step lasting 5 min at 72 °C.

The PCR products were then subjected to electrophoresis in a 1.5% agarose gel (w/v), stained with ethidium bromide for visualization. A 100 bp DNA ladder was used as a marker, and the identification of the *fsr*A, *fsr*B, and

*gel*E genes was confirmed by the visualization of amplification products measuring 474 bp, 428 bp, and 704 bp, respectively.

Statistical analysis

Experiments were performed in triplicates. All correlations were tested by the Pearson correlation coefficient. The association between the presence/absence of genes and different phenotypes was tested by Chi-Square test, for multiple categories (i.e., biofilm strength), or Fisher's Exact test, for two-category comparisons (i.e., gelatinase activity). *P*-value < 0.05 was considered significant. Visualization and statistical tests were performed in GraphPad Prism 9.5.0 (GraphPad, San Diego, CA).

Results

One hundred isolates were confirmed to be *Enterococcus* species based on their appearance as brownish-black colonies encircled by a black zone on bile esculin agar. Gram staining of these samples revealed Gram-positive coccobacilli, which were typically arranged in pairs or short chains. Additional identification procedures involved catalase and 6.5% NaCl tolerance tests. The results demonstrated that the isolates were catalase-negative and capable of growing in high concentrations of NaCl.

PCR identification of the isolates indicated that 93% of them were *E. faecalis* and 7% were *E. faecium*. All *E. faecium* isolates were excluded from further study.

The crystal violet assay, used to evaluate bacterial biofilm formation, showed that only four isolates did not form biofilm, while the majority were strong biofilm producers (n = 44; 47%). About 28% of the isolates were moderate biofilm producers (n = 26), and the remaining isolates were weak biofilm producers (n = 19; 21%; Fig. 1).

The nutrient gelatin plate method confirmed that 13 isolates had gelatinase activity, while the majority of the collected isolates (86%, n = 80) were unable to produce detectable gelatinase activity.

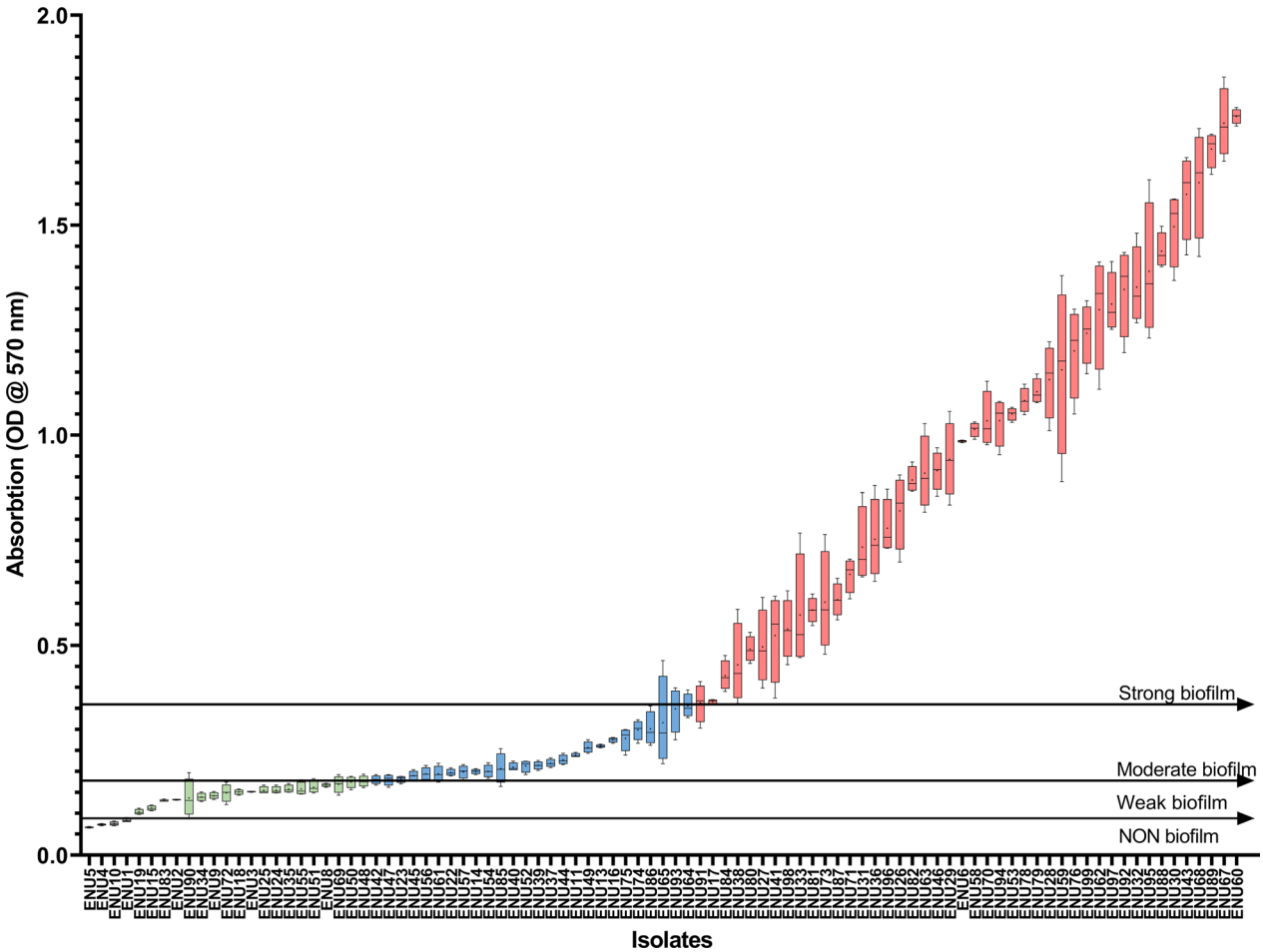

Fig. 1 Biofilm-formation by the tested *Enterococcus faecalis* isolates. The intensity of the biofilm formation by each clinical isolate is expressed as the absorbance of the dissolved dye from stained biofilms at 570 nm. The horizontal lines represent the absorbance cutoff values for non-, weak, moderate, and strong biofilm formation

Using PCR allowed the detection of the *fsr*A and *fsr*B genes in 24 and 26 isolates, respectively (26% and 28% of the isolates, respectively, Fig. 2).

No significant correlation was found between the intensity of biofilm formed by different isolates and the PCR detection of quorum sensing genes *fsr*A ($r=-0.14$), and *fsr*B ($r=-0.11$), or the gelatinase-encoding gene *gel*E ($r=-0.04$). Additionally, the intensity of the formed biofilms was not correlated with the gelatinase activity ($r=-0.06$; Fig. 3).

The *gel*E gene was detected in all gelatinase-positive isolates, and 50% (n = 40) of gelatinase-negative isolates, with a significant association ($p=0.0004$; Table 2), but weak positive correlation ($r=0.35$; Fig. 3).

The *fsr*A gene was detected in all gelatinase-positive isolates and 14% (n = 11) of gelatinase-negative isolates, while the *fsrB* gene was present in all gelatinase-positive isolates and in 16% (n = 13) of the gelatinase-negative isolate.

The PCR detection of *fsr*A and *fsr*B was positively and significantly correlated with gelatinase production ($r=0.68$ and 0.65, respectively, $p<0.0001$; Fig. 3 and Table 2).

The molecular detection of the *fsr*A, *fsr*B, and *gel*E genes together was directly correlated with the gelatinase activity ($r=1$). Likewise, the presence of just *fsr*A and *fsr*B together was directly correlated with the gelatinase activity ($r=1$; Fig. 3).

Discussion

In this study, 100 clinical enterococcal isolates were collected from patients with urinary tract infections and identified to the species level, where most of the isolates were *E. faecalis* (n = 93). A similar predominance of *E. faecalis* infection was reported in Egypt [18] and worldwide [19]. Most of the tested *E. faecalis* isolates (96%) were capable of biofilm formation to different intensities; only four isolates were non-biofilm former. Similar results about biofilm formation capabilities in *E. faecalis* are available [20]. In accordance with previous studies [21, 22], most of the isolates formed biofilms with either strong or moderate intensity (72%).

Only 14% of the tested isolates had detectable gelatinase activity on gelatin-agar plates. The low frequency of detected gelatinase activity in *E. faecalis* isolates was previously reported [23, 24]; however, in a study by Robert and colleagues (2004), the percentage of gelatinase-producing *E. faecalis* isolates from clinical and community settings reached 67% [25].

It is worth noting that available information about the role of the *fsr* locus in biofilm formation and intensity is still contradictory. Here, a lack of correlation was observed between the *fsr* locus presence and biofilm intensity, and similar results were previously reported [26]. To the contrary, other studies reported reduced biofilm formation among *fsr* mutants [27].

A direct correlation was observed in this study between the presence of the *fsr*A and *fsr*B genes and the gelatinase activity, and the presence of both genes together with the *gel*E genes was detected in all isolates with positive gelatinase activity. The correlation between the Fsr system and gelatinase activity was previously reported [3]; however, a study by Hashem et al. [21] reported *fsr*B as a stronger predictor of gelatinase activity.

The gelatinase activity was suggested to affect biofilm production and to be important for pathogenicity in different infection models [28]. Here, we did not find any correlation between gelatinase production and the intensity of biofilm formation. Similar results about the lack of correlation between biofilm intensity and gelatinase

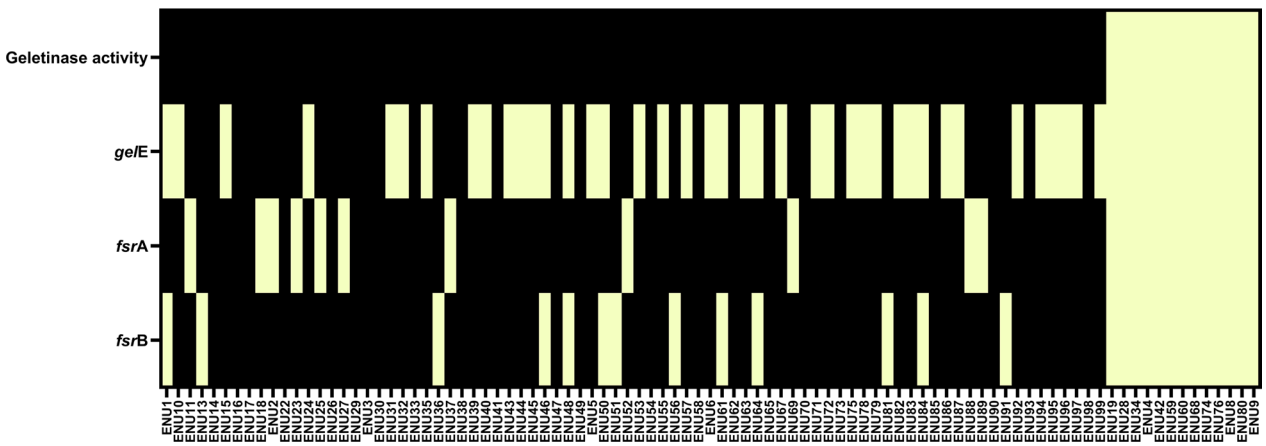

Fig. 2 The gelatinase activity, and the detectability of *gel*E, *fsr*A, and *fsr*B genes in the tested *Enterococcus faecalis* isolates. (Yellow: Present; black: Absent)

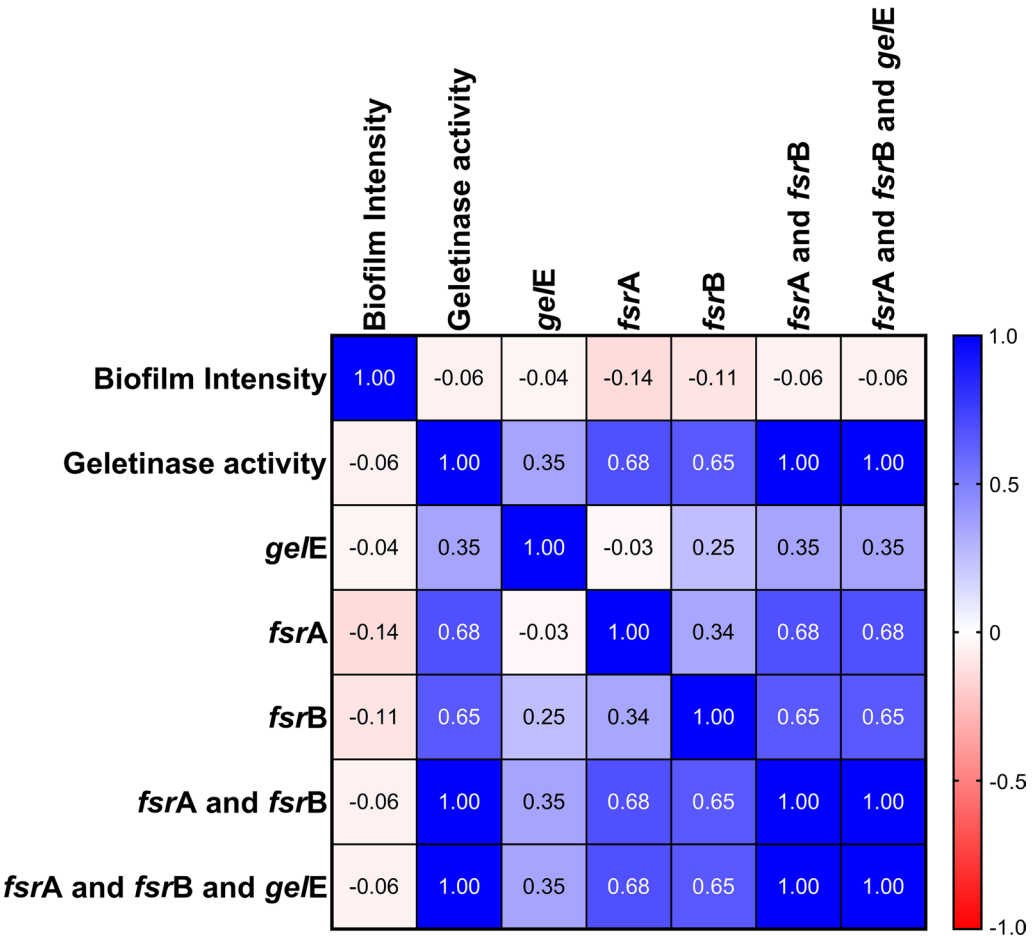

Fig. 3 The correlation between the detectability of *fsr*A, *fsr*B, and *gel*E genes, the gelatinase activity, and the intensity of formed biofilm. The correlation was estimated by the Pearson correlation coefficient. The color scale on the right represents the correlation coefficient

production in *E. faecalis* isolates were reported [21, 29]. On the other hand, some studies on *gel*E mutant strains confirmed the role of gelatinase production in biofilm formation [30, 31]. Therefore, further studies are needed to determine the exact role of gelatinase in biofilm production.

In this study, *gel*E gene was detected in 57% of collected isolates. However, gelatinase activity was only found in 14% (n = 13) of the isolates. This may have resulted from the partial deletion in the *fsr* locus as previously discussed by Qin et al. [32], who found that gelatinase activity was abolished after the deletion of the *fsr* locus. This loss of activity was documented [3].

In terms of diagnostic value of the *fsr* locus genes as biomarkers for gelatinase production, the finding that both *fsr*A and *fsr*B were positively correlated with the gelatinase production phenotype suggests that, while the molecular detection of these two genes is not fully correlated with the gelatinase phenotype, it still might be useful for rapid molecular screening or when culturing the bacteria is not possible. For example, in microbiome analysis studies, the detection of these genes in DNA extracted from fecal specimens or sewage samples could suggest potential for gelatinase activity.

Conclusions

The gelatinase activity of *E. faecalis* clinical isolates is strongly positively correlated ($r = 1$) with the presence of the quorum sensing-associated *fsr*A and *fsr*B genes, and no gelatinase activity was measurable when *fsr*A and *fsr*B were absent. Although reported in many studies, no direct correlation was found between *fsr*A, *fsr*B, or gelatinase production and biofilm formation intensity, which suggests that other genetic factors are associated with biofilm intensity.

Table 2 Association of gelatinase production, biofilm production, and the detectability of *fsr*A, *fsr*B, and *gel*E genes

Gelatinase production genotype	Number of isolates		*P* value (Fisher exact test)
	Positive	Negative	
*fsr*A+	13	11	< 0.0001
*fsr*A−	0	69	
*fsr*B+	13	13	< 0.0001
*fsr*B−	0	67	
*gel*E+	13	40	0.0004
*gel*E−	0	40	

Biofilm intensity genotype	Number of isolates				*P* value (Chi-Square test)
	Strong	Moderate	Weak	Non	
*fsr*A+	9	6	8	1	0.3344 (NS*)
*fsr*A−	35	20	11	3	
*fsr*B+	11	6	6	2	0.6658 (NS)
*fsr*B−	33	20	13	2	
*gel*E+	26	11	12	4	0.1283 (NS)
*gel*E−	18	15	7	0	

* *NS* not significant ($p \geq 0.05$)

Abbreviations

Fsr Fecal *Streptococcus* regulator
PCR Polymerase chain reaction
TSB Trypticase soy broth
UTI Urinary tract infection

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Not applicable

Author contributions
YAH conceived the study. MTK, RKA, and YAH designed experiments. KAA and YAH performed laboratory experiments. KAA, MTK, and RKA analyzed the data. KAA generated all figures. KAA and YAH drafted the manuscript; MTK and RKA revised the first drafts; all authors revised and agreed with the final version of the manuscript.

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Availability of data and materials
All data that support the findings of this study are provided in the manuscript. Raw data are available from the corresponding author, upon request.

Declarations

Ethics approval and consent to participate
The study was approved by the safety and ethics committee of the Faculty of Pharmacy, Cairo University, with approval number (MI 2634 for the year 2020).

Consent for publication
As no human subjects were involved in this work, no consent for publishing the results was needed.

Competing interests
The authors declare that they have no competing interests.

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