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Research Article

Characteristics of Enterococcus Faecalis in Periodontitis and Secondary Root Canal Infections in Northern Jordanians-A Pilot Study

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Abstract: Background: Enterococcus faecalis has been found to be related to periodontitis and secondary endodontic infections. In this study, we aimed to study samples of *E. faecalis* to determine their incidence and virulence in Jordanian patients. Methods: A total of 167 samples were collected from patients with periodontitis and secondary endodontic infections. The Kirby-Bauer method was used to determine the antimicrobial susceptibility. Biofilm formation was studied using the microtiter plate assay and congo red agar assay. Polymerase chain reaction (PCR) was used to detect the presence of *E. faecalis* virulence genes, namely; *asa1, gelE*, *cylA*, *esp*, *hyl*, *efaA*, and *ace*. Gelatinase and cytolysin activity were also tested using phenotypic methods. Results: Twenty-three (13.8%) samples were positive for *E. faecalis*. The highest resistance rates were observed against ampicillin (87%), followed by penicillin (82.6%), and erythromycin (60.9%). The highest susceptibility was for levofloxacin (100%), followed by gentamycin and chloramphenicol, each with 95.7%. Most isolates were able to produce biofilm (78.3%). Gelatinase and cytolysin activity were detected in 21.7% and 56.5% of isolates, respectively. *efaA* was significantly associated with *asa1* and *gelE* ($P < 0.05$), and *esp* was significantly associated with *cylA*, *gelE*, *ace*, *efaA* and *asa1* ($P < 0.05$). In addition, the *gelE*. gene was significantly associated with gelatinase production ($P < 0.01$). Conclusion: In conclusion, we have shown that *E. faecalis* is involved with periodontal disease and secondary root canal infection with several virulence genes detected. Clinical Relevance: Around 13% of the periodontitis and secondary root canal infection patients were positive for *E. faecalis* and isolates were resistant to commonly used antibiotics.

Keywords: oral microbiology, antimicrobial susceptibility, periodontics, endodontics

Abbreviations

PCR	Polymerase chain reaction
AS	Aggregation substance
CLSI	Clinical & Laboratory Standards Institute
NS	Non-susceptible

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DNA Deoxyribonucleic acid

1. Introduction

Enterococci are Gram-positive, facultative anaerobic cocci, that can resist harsh environmental conditions and persist for long periods [1], they are common inhabitants of the gastrointestinal and genitourinary tracts of humans and animals, but they can also be detected in soil, water, plants, and food [2]. Enterococci were initially considered as non-virulent, but they began to emerge as a leading cause of multidrug-resistant nosocomial infections in the 1970s and 1980s. Enterococci can cause many infections including bloodstream infections (septicemia), urinary tract infections, wound infections, meningitis, and infective endocarditis [3]. Among Enterococcus species, Enterococcus faecalis is the most predominant, accounting for up to 80-90% of enterococcal infections in humans [4].

Although *E. faecalis* is not a common colonizer of oral cavities with healthy dentition [5], it has been found to be associated with oral mucosal lesions in immunocompromised patients, periodontitis, peri-implantitis and endodontic (root canal) infections [6], Endodontic infections can be classified as primary and persistent; primary endodontic infections are usually polymicrobial and dominated by obligate anaerobic bacteria with the presence of some facultative anaerobes such as streptococci, whereas persistent infections are usually caused by gram-positive facultative bacteria [7]. *E. faecalis* has been frequently recovered from persistent endodontic infections (with failed endodontic treatments) and it has been occasionally recovered from primary endodontic infections (no previous endodontic treatment with necrotic pulp) [8]. Moreover, *E. faecalis* has been recovered, from subgingival biofilms of periodontitis patients (47.8%) compared to controls (17.1%) [9]. According to another study by Chidambar et al, a higher frequency of *E. faecalis* was found in subgingival biofilms of periodontitis groups (41.7%) [10]. With evidence supporting the presence of *E. faecalis* in periodontitis and secondary endodontic infections, samples for this study were taken from these locations. *E. faecalis* has also been found to colonize dental implants and act as a co-factor in the polymicrobial process leading to peri-implantitis [11]. The objective of this study is to determine the prevalence and antimicrobial sensitivity of *E. Faecalis* obtained from periodontitis and secondary root canal infections in Northern Jordanians. We hypothesized that *E. faecalis* may play a role in both diseases.

E. faecalis is intrinsically resistant to many antimicrobial agents including beta-Lactams, Cephalosporins, Clindamycin and Aminoglycosides. Furthermore, it can acquire resistance to many classes of antimicrobial agents including Quinolones, Macrolides, Tetracyclines, and Glycopeptides [12]. Although the incidence of resistant *E. faecalis* strains is more common in nosocomial or systemic infections, resistance to antibiotics, commonly used in dentistry, is often detected in *E. faecalis* isolates form endodontic infections and periodontitis [7]. so periodic and accurate antimicrobial susceptibility information of oral pathogens is necessary to guide the management of Peri-implantitis, Periodontitis and endodontic therapy, as well as calling attention to the problem of antimicrobial resistance [9, 11].

The exact role of *E. faecalis* in the pathogenicity of endodontic infections and periodontitis is still uncertain. *E. faecalis* has many virulence factors that mediate its adhesion, colonization, biofilm formation, invasion into the host tissues, and modulation of the host immunity. These factors include cytolysin (*cyl*, beta-hemolysin), proteolytic enzymes (gelatinase (*gelE*) and serine protease), adhesins (aggregation substance (*AS*), enterococci surface protein (*esp*), collagen adhesion protein (*ace*), *E. faecalis* endocarditis antigen A (*efaA*)), Hyaluronidase (*hyl*) and capsular and cellular wall polysaccharides [13]. Another important property of *E. faecalis* is its ability to form biofilms in untreated and treated teeth which make it more resistant to chemicals and antibiotics used in periodontal and endodontic treatment. Furthermore, biofilms facilitate the spread of resistance and virulence genes to other species via gene exchange [14]. In addition to possessing various resistance and virulence genes, *E. faecalis* is able to share these traits among species via Horizontal gene transfer. So, the presence of *E. faecalis* in dental diseases might make the oral cavity a reservoir for virulent and resistant strains [15].

In the current study, antimicrobial susceptibility testing, biofilm formation capacity, and incidence (occurrence) of virulence genes (*gelE*, *ace*, *efaA*, *asa1*, *cylA*, *esp*, and *hyl*) of *E. faecalis* bacterial samples isolated from Jordanian patients with Periodontitis and secondary root canal infections were evaluated.

2. Materials and methods

2.1 *Sample collection*

Study subjects that were included have all been cleared of specific medical conditions that may affect periodontal status. Subjects were excluded if they had any of the following medical conditions/diagnoses: diabetes, Chediak-Higachi syndrome, pregnancy, and any form of immunosuppression. In addition, subjects who had any periodontal treatment or prescribed antibiotics in the last three months prior to sample collection were not included in the study. Secondary endodontic infections were determined by an endodontic specialist based on clinical and radiographic findings, persistent periapical radiolucencies, and voids in root canal filling along with persistent symptoms were considered reasons for retreatment (endodontic failures). Periodontitis patients were examined by a periodontist to determine the stage and grade of periodontitis based on the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions, patients with stages ranging from 2-4 were included in the study presenting with different grades (A-C) [16].

Samples were collected using sterile paper points inserted to the full length of the root canal and kept in place for 60 seconds to absorb root canal contents. While in periodontitis patients the subgingival plaque was collected using a sterile site-specific gracey curette, after which the plaque was removed from the curette with sterile paper points. Paper points containing the sample were then immediately spread on a plate of bile-esculin azide agar (Oxoid, United Kingdom) and transferred to the incubator.

2.2 *Sample identification*

All collected samples were sub-cultured on blood agar, at 37 °C for 24 hours. For each isolate, 3 to 4 fresh colonies were inoculated in 3 mL of nutrient broth at 37 °C for 18 hours. Then, 500 µl of each broth was mixed with 500 µl of 50% of sterile glycerol. All pure bacterial-glycerol stocks were stored at -80 °C for further testing. The test scheme of Facklam and Sahm was used for identification at a genus and species level [17].

Briefly, to test the isolates at a genus level, bile esculin azide agar was used and blacking of the media indicated a positive result, Sodium Chloride tolerance was also used to test the genus of the isolates using Brain Heart Infusion media (Oxoid, United Kingdom), supplemented with 6.5% NaCl (w/v), and turbidity of medium after 24 h incubation at 37 °C indicated a positive result.

To identify the species of the isolate, arabinose fermentation was tested using brain heart infusion broth (Oxoid, United Kingdom) supplemented with 1% arabinose and phenol red as an indicator, *E. faecalis* cannot ferment arabinose, therefore the media stays red indicating a positive result. The ability to reduce tellurite was tested using Todd-Hewitt broth media (Oxoid, United Kingdom) supplemented with 0.04% potassium tellurite (Himedia, India); *E. faecalis* strains have the ability to reduce tellurite and blacking of the media indicated a positive result. Utilization of pyruvate was tested using a commercially available kit (Hardy Diagnostic, California), 1% pyruvate broth tubes were used, since *E. faecalis* strains can utilize pyruvate and produce acid, which turns the broth into a yellow color (positive result).

2.3 *Antimicrobial susceptibility testing*

The Kirby-Bauer disk diffusion method was used on Muller Hinton agar to evaluate the antimicrobial susceptibility profile of isolates to fourteen antimicrobial agents. The selection of antimicrobial agents and interpretation of results were done according to the Clinical and Laboratory Standards Institute (CLSI) Performance Standards for Antimicrobial Susceptibility Testing [18].

2.4 *Gelatinase and cytolysin activity testing*

Gelatinase production was tested through detection of gelatinase activity on nutrient gelatin medium (Oxoid, United Kingdom), liquefication of the medium indicated a positive result for gelatinase production [19]. Cytolysin production was tested through the detection of cytolysin activity (hemolysis) on blood agar (Oxoid, United Kingdom); a clear zone around the colonies indicated positive cytolysin production [20].

2.5 *Detection of biofilm formation*

Biofilms were detected qualitatively using the Congo red agar method (black colonies with a dry crystalline consistency suggesting biofilm formation) [21], and quantitatively by the biofilm microtiter plate assay [22].

2.6 *Detection of E. faecalis virulence genes*

Multiplex PCR was used to detect genes (*asa1*, *gelE*, *cylA*, and *esp*). Primer sequences and product sizes are listed in Table 1. All primers were obtained from Macrogen (Seoul, South Korea). A conventional PCR was performed to detect virulence genes (*hyl*, *efaA*, and *ace*) as previously described [13, 23-25].

Table 1. Primers were used for PCR in this study

Gene	Primer sequence (5′-3′)	Amplicon size (Product size) [b.p]
asa1	F-GCACGCTATTACGAACTATGA R-TAAGAAAGAACATCACCACGA	375
gelE	F-TATGACAATGCTTTTTGGGAT R-AGATGCACCCGAAATAATATA	213
cylA	F-ACTCGGGGATTGATAGGC R-GCTGCTAAAGCTGCGCTT	688
esp	F-AGATTTCATCTTTGATTCTTGG R-AATTGATTCTTTAGCATCTGG	510
hyl	F-ACAGAAGAGCTGCAGGAAATG R-GACTGACGTCCAAGTTTCCAA	276
efaA	F-GCCAATTGGGACAGACCCTC R-CGCCTTCTGTTCCTTCTTTGGC	688
ace	F-GGAATGACCGAGAACGATGGC R-GCTTGATGTTGGCCTGCTTCCG	616

3. Results

A total of 167 samples were collected across two categories, 126 samples were collected from periodontitis patients and 41 samples were taken from secondary endodontic infections. 23 samples were identified as *E. faecalis*, and the overall prevalence was 13.7% (Table 2). Considering individual categories, we found a prevalence of 13.49% for samples isolated from periodontitis patients and a prevalence of 14.63% for samples isolated from endodontic failures.

Table 2. Prevalence of *E. faecalis* in this study

	Source	
	Periodontitis	Endodontic failures
Count	17	6
Percentage of samples	73.9%	26.1%
Prevalence	13.49%	14.63%

3.1 *Antimicrobial susceptibility results*

The antimicrobial susceptibility results of *E. faecalis* to several antimicrobial agents, based on CLSI 2017 recommendations, are listed in Table 3. The resistant (R) and intermediately susceptible (I) isolates were grouped together into one group, non-susceptible (NS). The highest rates of resistance were observed against ampicillin (87.0%), followed by penicillin (82.6%), erythromycin (60.9%) and Quinupristin-dalfopristin (56.5 %). Antimicrobial resistance percentages are shown in Figure 1. In contrast, the highest rates of susceptibility were observed against levofloxacin (100%), followed by chloramphenicol (95.6%), gentamicin (95.6%), ciprofloxacin (82.60%), teicoplanin (82.6%), rifampin (26.10%), fosfomycin (13%) and vancomycin (17.40%) (Figure 1).

Table 3. Antimicrobial susceptibility of *E. faecalis* reported as resistant (R), susceptible (S), intermediately susceptible (I) and non-susceptible (NS)

Antimicrobial agent	R		S		I		NS*	
	Count	Row N%	Count	Row N%	Count	Row N%	Count	Row N%
Penicillin	19.0	82.6	4.0	17.4	0.0	0.0	19	82.609%
Ampicillin	20.0	87.0	3.0	13.0	0.0	0.0	20	86.957%
Vancomycin	5.0	21.7	14.0	60.9	4.0	17.4	9	39.130%
Teicoplanin	3.0	13.0	19.0	82.6	1.0	4.3	4	17.391%
Erythromycin	14.0	60.9	8.0	34.8	1.0	4.3	15	65.217%
Tetracycline	10.0	43.5	13.0	56.5	0.0	0.0	10	43.478%
Levofloxacin	0.0	0.0	23.0	100.0	0.0	0.0	0	0.000%
Ciprofloxacin	0.0	0.0	19.0	82.6	4.0	17.4	4	17.391%
Nitrofurantoin	5.0	21.7	17.0	73.9	1.0	4.3	6	26.087%
Rifampin	5.0	21.7	12.0	52.2	6.0	26.1	11	47.826%
Chloramphenicol	0.0	0.0	22.0	95.7	1.0	4.3	1	4.348%
Quinupristin-dalfopristin	13.0	56.5	10.0	43.5	0.0	0.0	13	56.522%
Gentamicin	1.0	4.3	22.0	95.7	0.0	0.0	1	4.348%
Fosfomycin	2.0	8.7	18.0	78.3	3.0	13.0	5	21.739%

*NS includes both intermediately susceptible and resistant isolates

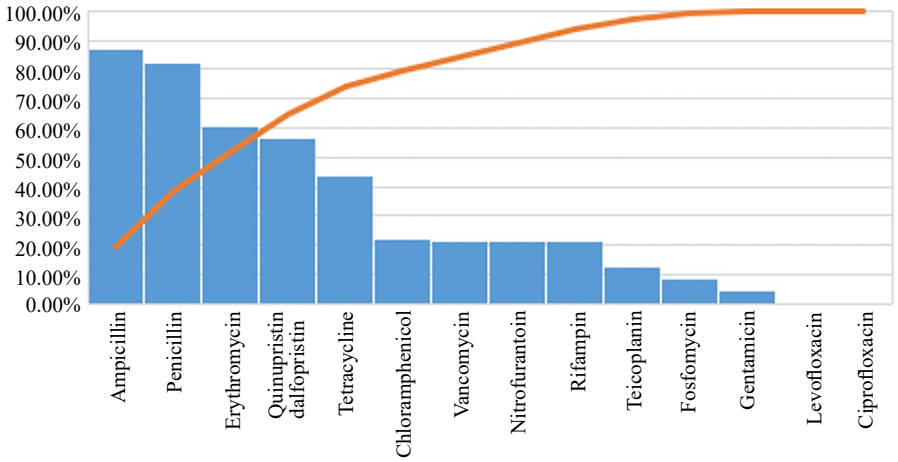

Figure 1. Antimicrobial resistance in *E. faecalis* against several antimicrobial agents, the Kirby-Bauer method was used to determine resistance of *E. faecalis* against antimicrobial agents, this bar graph shows the results from highest resistance (Left) to lowest resistance (Right), the orange line signifies the susceptibility of *E. faecalis* against antimicrobial agents from lowest susceptibility (Left) to highest susceptibility (Right)

3.2 *Biofilm formation capacity assays of E. faecalis isolates*

The ability of the isolates to produce biofilm was measured using two methods, the congo red agar test (qualitative) (Table 4), and the biofilm microtiter plate assay (quantitative) (Table 5). No significant associations were found between antimicrobial susceptibility profile and biofilm formation capacity ($P > 0.05$) (Figure 2).

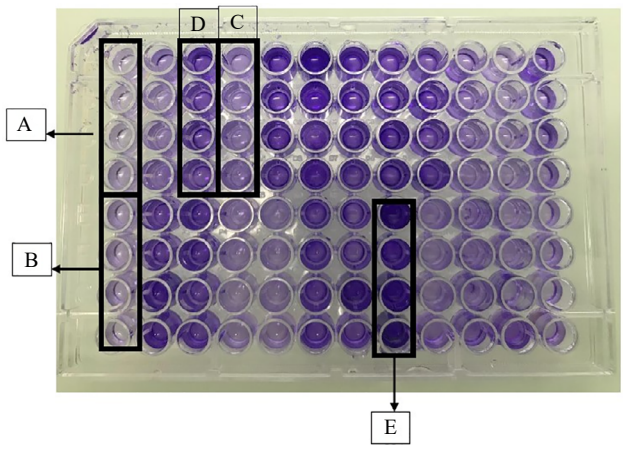

Figure 2. Image of a representative biofilm formation assay microtiter plate after using a micro-ELISA autoreader at a wavelength of 570 nm. A: Negative control, B: Non-biofilm producer, C: Weak biofilm producer, D: Moderate biofilm producer, E: Strong biofilm producer

Table 4. Congo red agar

		Count	Row N%	Cumulative percent
Valid	Negative	9	39.1	39.1
	Positive	14	60.9	100.0
	Total	23	100.0	-

*A positive biofilm-formation result was indicated by development of black colonies on congo red agar plates, while nonproducing strains appeared as red colonies

Table 5. Microtiter plate assay

	Count	Row N%
Non-biofilm producer	5	21.7
Weak biofilm producer	5	21.7
Moderate biofilm producer	4	17.4
Strong biofilm producer	9	39.1
Total	23	100.0

The Optical Density (OD) of each well was measured with a micro-ELISA auto-reader at a wavelength of 570 nm average OD values were calculated for all tested isolates and negative controls. The optical density cut-off (ODc) value which separates biofilm forming from non-biofilm forming strains was calculated for each microtiter plate separately. ODc was defined as three standard deviations (SD) above the mean OD of the negative control: ODc = average OD of negative control + 3 × SD of negative control. The isolates were categorized as the following:

• Non-biofilm producer: OD ≤ ODc

• Weak biofilm producer: ODc < OD ≤ 2 × ODc
• Moderate biofilm producer: 2 × ODc < OD ≤ 4 × ODc
• Strong biofilm producer: OD > 4 × ODc

3.3 *Gelatinase and cytolysin testing*

Results of Gelatinase and Cytolysin activity are shown in Table 6, Among the *gelE*-positive isolates, 50% did not show gelatinase activity. One isolate had gelatinase activity despite being negative for *gelE*. *gelE* was significantly associated with gelatinase production ($P < 0.01$). Among the *cylA* positive isolates, 100% were positive for cytolytic activity; ten *cylA* negative isolates (76.9%) demonstrated hemolytic activity. No significant association was found between the presence of *cylA* gene and cytolysin production ($P > 0.07$).

Table 6. Gelatinase and Cytolysin activity results

		Count	Row N%	Cumulative Percent
Gelatinase	Negative	18	78.3	78.3
	Positive	5	21.7	100.0
	Total	23	100.0	-
Cytolysin	Negative	10	43.5	43.5
	Positive	13	56.5	100.0
	Total	23	100.0	-

3.4 *Virulence genes of E. faecalis isolates*

The distribution of virulence genes among *E. faecalis* isolates is shown in Table 7. *GelE* and *ace* were the most frequently detected genes (34.8%), followed by *efaA* and *asa1* (26.1%), and *cylA* (13%). Figure 3 shows representative gel electrophoresis for the detection of *cylA*, *esp*, *gelE* and *asa1* genes following multiplex PCR (Figure 3).

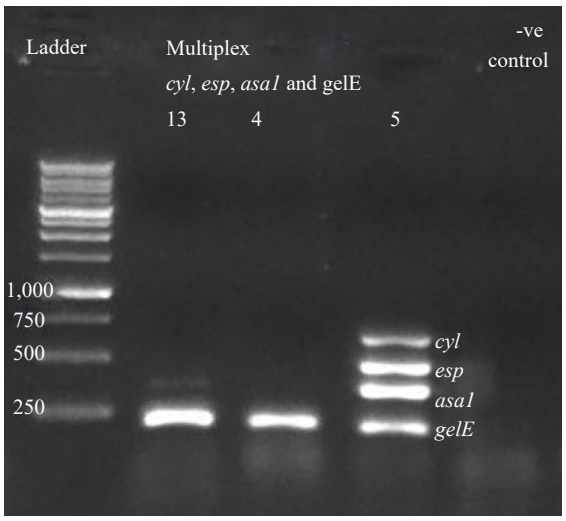

Figure 3. Image of a representative gel for detection of *esp*, *gelE*, *cylA*, and *asa1* using multiplex PCR results samples number 13, 4 and 5 were used as a demonstration: 5 µl of the PCR product were analyzed using 2% agarose gel electrophoresis stained with ethidium bromide at 150 volts for 45 min, a 1 kb DNA ladder Genedirex was used for size selection (determination): Samples demonstrating *gelE* (213 bp), *asa1* (375 bp), *esp* (510 bp), and *cylA* (688 bp)

Table 7. Distribution for virulence genes among *E. faecalis* isolates

Virulence gene		Count	Row N%
efaA	Negative	17	73.9
	Positive	6	26.1
	Total	23	100.0
cylA	Negative	20	87.0
	Positive	3	13.0
	Total	23	100.0
esp	Negative	21	91.3
	Positive	2	8.7
	Total	23	100.0
asa1	Negative	17	73.9
	Positive	6	26.1
	Total	23	100.0
gelE	Negative	15	65.2
	Positive	8	34.8
	Total	23	100.0
ace	Negative	15	65.2
	Positive	8	34.8
	Total	23	100.0
hyl	Negative	22	95.7
	Positive	1	4.3
	Total	23	100.0

4. Discussion

The prevalence rate of *E. faecalis* in periodontitis patients in this study was 13.49%, which is comparable to a study by Rams in 1992 in which the prevalence rate of *E. faecalis* in chronic periodontitis patients was 5.1% [26]. In contrast, Souto reported a higher prevalence rate (47.8%) when studying Brazilian populations in 2008 [9].

A large range of prevalence rates have been reported in previous studies of endodontic infections. Barbosa-Ribeiro et al. found *E. faecalis* in 100% of the root canals they investigated [27], whereas Endo et al. reported a prevalence of 23.3% (8). In the current study, we found a relatively smaller prevalence of 14.63%.

Resistance to antimicrobial agents has been rising in *E. faecalis* strains specifically in clinical isolates, they are intrinsically resistant to β-lactams and low-level aminoglycosides [12]. Furthermore, a hypothesis suggesting that horizontal transfer of antimicrobial resistance genes can occur between Streptococcus gordonii and *E. faecalis* in root canals has been confirmed, which creates the need for further investigation of the polymicrobial biofilms in root canals and the interplay of horizontal gene transfer and endodontic infections [28].

Regarding antimicrobial susceptibility results of other studies in comparison to the present study, our results of antibiotic susceptibility contradict with Chi et al. who found that erythromycin and tetracycline are capable of completely eradicating *E. faecalis* from oral biofilms. However, oral *E. faecalis* isolates in this study showed high to moderate resistance to erythromycin (60.9%) and tetracycline (43.5%), respectively [29]. In another report by

Anderson, high levels of erythromycin resistance were reported by samples from food, oral sites, and clinical samples [19]. In contrast to the present study, Pinheiro et al. reported endodontic isolates of *E. faecalis* to be highly susceptible to penicillin (100%), which proved that isolates from different locales have different resistance traits, as the isolates in Brazil were less likely to produce *β*-lactamase making them more susceptible to penicillin, whereas the isolates in the current study were highly resistant to penicillins which could mean that their intrinsic resistance to penicillins is at least in part due to *β*-lactamase production [30].

Differences in antimicrobial profiles and the emergence of resistance in different geographical regions are common due to variations in antibiotic prescription practices, and over-prescription, in different countries. As the present study is one of the extensive investigations in our region to evaluate antimicrobial profiles and resistance profiles of oral isolates of *E. faecalis*, its findings must be further examined by exploring a much larger sample of oral enterococci. Until further data is available, it is prudent to observe the relatively high levels of resistance to commonly prescribed antibiotics in regular dental practices such as penicillin (82.6%) and tetracycline (43.5%) which may not be effective in eradicating oral enterococci and due to horizontal gene transfer, may not be effective in eradicating oral bacteria causing dental infections in general. The differences will make it necessary for regional antimicrobial studies to determine the most effective antimicrobial therapies for each region.

Table 8. *E. faecalis* virulence genes in different studies

Study Virulence gene	Anderson 2016 (Germany)	Aghdam 2017 (Iran)	Dahlen 2011 (Germany)	Barbosa-Ribiero 2016 (Brazil)	This study (Jordan)
gelE	99%	81%	10%	75%	34.80%
ace	-	85%	-	100%	34.80%
efaA	100%	82%	93.30%	95.00%	26.10%
asa1	92%	33%	96.70%	60.00%	26.10%
esp	70.50%	56.00%	93.30%	70.00%	8%
cylA	47.40%	0.00%	100%	0%	13%
hyl	0.00%	2.00%	16.70%	-	4.30%

Using the microtiter plate assay to quantitatively evaluate biofilm formation capacity of *E. faecalis* revealed that most of the isolates in our study were able to produce biofilm (78.3%) (Table 8). Additionally, most isolates were strong biofilm producers (39.1%), followed by weak and non-biofilm producers (21.7% for both). These results contrast with Duggan et al. whose results showed that only 11% of their endodontic and oral *E. faecalis* isolates were considered biofilm producers [31]. Our results appear to be in agreement with Mohamed et al. who reported 92% of their clinical isolates were biofilm producers (Strong 22%, moderate 56% and weak 14%) [32]. Wang et al. reported that 75.4% of their samples were biofilm producers which agree with our results [33].

The particularly complex surface topography of dentin in root canals which have rough surface irregularities where the bacteria is present in dentinal tubules filled with necrotic tissue differs from the sterile circumstances found in the microtiter plates used in this study. In addition, periodontal and endodontic infections alike are polymicrobial, and it has been demonstrated that heterogenous colonies have a definite advantage over homogenous ones in the ability to produce surface biofilms [34].

The association between biofilm production and resistance determinants in bacteria has been extensively studied. In this study, our results showed no association between biofilm formation capacity and antimicrobial susceptibility testing ($P > 0.05$). Our results were in agreement with a study carried out by Avila-Novoa et al. who did not find any clear association between biofilm production and susceptibility. The study of Cepas et al. included various Gram-negative

bacteria (E. coli, K. pneumoniae, and P. aeruginosa), which also found no direct association between possessing multidrug-resistant phenotype and biofilm production. In a study carried out by Qi et al. 2016, in typing of A. baumannii isolates, they revealed that non-multidrug resistant isolates were more common in non-biofilm producers [35-37].

This is in contrast with Dale et al. who found evidence suggesting that *E. faecalis* genetic determinants can facilitate antimicrobial resistance within biofilms [38]. As well as Kafil et al. *concluded* that the presence of *esp* as an indicator of biofilm formation in *E. faecalis* resulted in the increased incidence of antimicrobial agent resistance. Interestingly, another study by Kafil found that some antibiotics such as gentamycin can upregulate the expression of genes like *efaA* resulting in an increase of biofilm formation which in turn may result in higher antimicrobial resistance [39].

When using classic and multiplex PCR to detect *E. faecalis* virulence genes, we found that *gelE* and *ace* were the most frequently detected genes (most prevalent) (34.8%), followed by *efaA*, *asa1* (26.1%) and *cylA* (13%) (Table 8). Our results differ from those of Anderson et al. who reported 99% of their samples possessed the *gelE* gene and 92% had the *asa1* gene [19]. An earlier study by Dahlen et al. reported a lower *gelE* percentage in oral *E. faecalis* isolates (10%) [40]. Aghdam et al. reported similar results in which *ace* was the most prevalent gene (85%) followed by *efaA* (82%) [41], as well as the results reported by Barbosa-Ribiero in 2016 which revealed that ace was most prevalent (100%), followed by *efaA* (95%) and *gelE* (75%) [27].

In the present study, no significant associations were found when the relationship between biofilm formation capacity and the presence of *E. faecalis* virulence genes was evaluated ($P > 0.05$). This was the case in a study by Duggan et al., where they found no relation between biofilm formation ability and virulence genes *asa1*, *cylA*, *esp* and *gelE* [31], suggesting that *E. faecalis* biofilm formation was affected by more than one virulence gene. Therefore, fluctuations in the expression of said genes would not affect the biofilm formation capacity of the isolate severely [33]. This also implies that extrinsic and intrinsic factors may influence the production of biofilms and the expression of biofilm genes [42].

Phenotypic assays were used to evaluate gelatinase and cytolytic activity of isolates, gelatinase activity was observed in 21.7% of the isolates, whereas hemolysis (cytolysin activity) was detected in 56.5% of the isolates [43]. Isolates in the current study had more gelatinase and cytolysin activity than those of Dahlen et al. in which gelatinase activity was observed in 10% of isolates while hemolysis was observed in 16.7% [40]. Sedgley et al. reported hemolysis and gelatinase activity in 36.3% of the sample [44]. Whereas Aghdam et al. 2017 reported gelatinase activity in 77% of his samples [41].

Interestingly, among the *gelE*-positive isolates, 50% were unable to degrade gelatin possibly indicating a silent gelE gene, the reason behind this might be the lack of the 23.9 kb region of the *fsr* locus [45]. One isolate had gelatinase activity despite being negative for gelE which is an interesting finding that may be explained by point mutations in the primer binding regions of these genes which could affect the primer binding thus preventing PCR amplification. Among the *cylA* positive isolates, 100% were non-hemolytic on blood agar (no cytolysin production), also possibly indicating a silent *cylA* gene.

Ten *cylA* negative isolates (76.9%) demonstrated hemolytic activity which contradicts the expectation that also might indicate point mutations in the primer binding regions in the genes affecting the PCR amplification, this possible problem could be solved by using primers that bind to different regions of these genes. gelE and cylA positive strains that showed no gelatinase or cytolysin production could also indicate the involvement of other genes in the expression control of these genes [46]. The *gelE* was significantly associated with gelatinase activity ($P < 0.01$). No significant association was found between the presence of *cylA* gene and cytolysin production ($P > 0.7$).

Interpretation of the results of this present study suggests that while *E. faecalis* is an important causative factor of nosocomial infections, it does not appear to be as prevalent in this sample of Jordanian patients with periodontitis and secondary endodontic infections. Moreover, the low presence of virulence genes in comparison to studies from different regions may suggest that isolates used in this study are less virulent and are therefore not as challenging to eradicate or do not play an important part in oral infections, namely periodontitis and endodontic infections, in the Jordanian population.

Further studies are needed to evaluate the significance of *E. faecalis* as an oral infectious agent and as a nosocomial infectious agent with larger sample sizes (the present study includes a relatively small number of isolates) and with a geographic distribution across Jordan.

5. Conclusion

Significant associations were found between antimicrobial susceptibility profile results and the presence of numerous E. faecalis virulence genes. Due to the sample size, further studies are needed to evaluate the significance of E. faecalis as an oral infectious agent and as a nosocomial infectious agent and with a geographic distribution across Jordan.

Contributing authors

Haytham Qinawi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing-Original draft, Visualization, Project administration.

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Conflict of interest

The authors declare no competing financial interest.

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