Sys Rev Pharm 2020; 11(6): 742 – 748
A multifaceted review journal in the field of pharmacy
E-ISSN 0976-2779 P-ISSN 0975-8453

Some Virulence Factors of *Enterococcus Faecalis* Isolated from Root Canal Infections Combined with Effect of Some Irrigation Solution against *E.Faecalis*

Hajir Hassan Hussein[1], Fatima Malik Abood[2], Ahmed Ghanim Alhelal[3]
[1]MSc. Oral Microbiology, college of Dentistry, University of Babylon, Babylon province, Iraq.
[2]PhD. Microbiology, college of Dentistry, University of Babylon, Babylon province, Iraq.
[3]PhD Endodontics college of Dentistry, University of Babylon, Babylon province, Iraq.
Corresponding author: Hajir Hassan Hussein
E-mail: msdrhager8769@gmail.com

Article History: *Submitted: 10.04.2020* *Revised: 14.05.2020* *Accepted: 22.06.2020*

ABSTRACT

Back ground: Root canal infections refer to any infection occur in the dental root canal caused by bacteria mostly anaerobes.
Objective: to isolate E. *faecalis* by cultivation technique and to investigate the virulence factors, also to study a relationship between *E. faecalis* isolated from root canal infected cases and age. Moreover studying effect of some irrigation solution against E. *faecalis*.
Results: the results of cultivation technique of current study was showed that which about (68.8% n=55) were positive cases, while, about ((31.3%, n=25) were negative cases, and 60% of isolates has ability to protease production while about 90% of *E.faecalis* has ability for production of gelatinase, and most isolates have ability of adherence to oral epithelial cell and 100% biofilm production. Finally there is statically significant difference between age group and *E. faecalis* isolated from endodontic infections.
Keywords: *E. faecalis,* Root canal infections, Biofilm, Adhesion
Correspondence:
Hajir Hassan Hussein
MSc. Oral Microbiology, College of Dentistry, University of Babylon, Babylon Province, Iraq
E-mail: msdrhager8769@gmail.com
DOI: 10.31838/srp.2020.6.109

INTRODUCTION

Enterococci (genus *Enterococcus,* former group D streptococci). *E.faecalis* is a Gram-positive bacteria, non-spore-forming, catalase-negative, ferments glucose, and fermentative. Moreover, they are a part of the habitual flora found in the mouth, facultative anaerobic cocci may be implicated as an opportunistic pathogen in different kinds of nosocomial infections in immune compromised patients (1, 2).

There is additional characteristics difference from other pathogens, including the capability to colonize in the root canal and survive for long period in latent phase then become infectious organism without the support of other bacteria, their Gram stain morphology can be in many forms including: non-motile, commensal, spherical bacteria. It appears in planktonic form, in pairs and chains. Their cells are ovoid and are about 0.5–1 μm in diameter, additionally, *E.faecalis* has unique characteristics, they can survive in extreme alkaline pH as high as 9.6 and high salt concentrations. It has been succeeded in competition with other microorganisms, by invading dentinal tubules and resist nutritional deprivation (3,4). *E. faecalis* is considered as the most commonly existed bacterial species that cause both primary and secondary root canal infections. In fact, the bacteria that are colonized inside the infected root canal are biofilm bacteria rather than free floating bacteria, which represented as heterogeneous aggregation of microbial cells that are embedded in a self-made extracellular polymeric substance matrix (EPS) (5,6,7).

The role of *E.faecalis* in the oral cavity has not yet been elucidated. *E.faecalis,* although not usually considered to be part of the normal oral microflora, has been found in common dental diseases such as: periodontitis, periimplantitis and dental caries. *E.faecalis* has been found primarily in secondary endodontic infections with a prevalence of 24% to 70% (8,9,10).

Although The presence of *E.faecalis* is associated with both a primary and a persistent endodontic infection. *E.faecalis* is isolated in 10% of the cases of a primary endodontic infection. According to some authors, it is more often found in asymptomatic cases than in symptomatic cases. Other studies have shown that *E.faecalis* is more often isolated in teeth with failed treatment within the range of between 30% and 90% (11,12,13).

E.faecalis have the ability to adapt to changing environment help it to survive in root canal and cause re-infection and have capability to adherence to epithelial cells and root canal wall and aggregation to form communities called biofilm because all theses traits it was called the microorganism's antimicrobial resistance (14,15,16).

One of important reason for the hardness of elimination of *E. faecalis* infections were virulence factors that are responsible of the pathogenicity. The important virulence traits of *E. faecalis* are cell surface-associated protein, namely, Enterococcal surface protein (ESP), secreted toxins such as cytolysin, haemolysin, gelatinase, aggregation substance (AS), protease and cell wall polysaccharide. (17).

The main goal of current study was isolated *E.faecalis* and investigated the virulence factor of *E.faecalis* isolated from endodontic infection by cultivation technique .as well as, to assess the effectiveness of antibacterial irrigation solutions on *E.faecalis.*

MATERIAL AND METHODS

Eighty samples were collected for the current study from patients attending the Conservative Dentistry dental clinics of Babylon University -college of dentistry and private dental clinics with an age range of (15-70 y).

Sample collection

Five sterile paper points were used to collect each sample from the pulp canal by insertion them individually inside the pulp, each paper point was retained in the pulp space for 30 seconds, then a sterile plain tube containing 5 ml of brain heart infusion broth was used to kept these paper points. After that, all the Samples were transported to a microbial laboratory within 4 hours, although immediate transfer was preferred whenever possible. Finally all tubes were retained in an anaerobic incubator at 35ºC for 72 hours until use (18).

Bacteriological study

In this study, all samples were collected by paper point send to bacteriological study which included multiple steps started with culturing on mitis slaveries agar and incubated at anaerobic condition at 37c for 48 hours then staining with gram stain and examination under the light microscope. After that, biochemical tests were utilized to detect presence of *E.faecalis*. Virulence factor testes were used to more detection of it, moreover, all these samples have been tested the effect of some irrigation solution on *E.faecalis* in vitro.

RESULTS

The current study included 80 subjects suffering from endodontic infections. The age range extend from (15- 70) years. *E.faecalis* was highly isolated from age group (11-30) years (45%), likewise to age group (31-50) years, while age group (51-70) was associated with low percentage (14%). The study show median age (33) with mean of age (*34.950*) and SD (*12.7248)* and *p –value* (0.04).

As table (1) shows, there is high significant difference (P = *0.04)* between the two groups, these values are highlighted by statically significant difference between age group and *E. faecalis* isolated from endodontic patients.

Table 1: Association of E. faecalis isolated from endodontic patients with age

Age (Binned group) (years old)	Positive bacteria N(%)	Negative bacteria N(%)	Total number (%)	*P VALUE
11-30	26(47%)	10(40%)	36(45%)	
31-50	25(46%)	8(32%)	33(41%)	
51-70	4(7%)	7(28%)	11(14%)	
Total number (%)	55(100%)	25(100%)	80 (100%)	0.042
*Pearson chi Square	***s* significant less than ≤ 0.05***			

As can be observed from the table (2), the light microscopic examination and biochemical test showed that all isolates have the capability to cultivate on selective media (mitis slaveries agar) at anaerobic condition, in which, the colony morphology were appeared, blue-black color, shiny, slightly raised, While the colony appearance on blood agar showed no hemolytic, circular, convex colonies with entire margin, Gram staining, positive Cocci. Additionally, the appearance under the microscope revealed either cocci or coccobacilli.

The biochemical test utilized to assure the identification of *E.faecalis* isolates illustrated that all isolates were positive to growth in 6.5% NaCl, bile esculin, ferment the following sugar, glucose, fructose, maltose, sucrose, while the negative tests appear in catalase, oxidase, indole, simmon citrate, and there were variable results appeared in gelatin hydrolysis and hemolysis, variable from (Alfa to Gama).

Table 2: Microscopic examination and biochemical test of enterococcus faecalis

Tests	Results
Characteristics	
Growth on selective media (Mitis Salivarius Agar)	+ve
Colony morphology of Mitis Salivarius Agar	Blue-black, shiny, and slightly raised colonies
Shape under light microscope	Cocci ,coccobacilli
Gram stain	+ve
Colony morphology on blood agar	non hemolytic ,circular ,convex colonies
Catalase	-ve
Oxidase	-ve
Indole	-ve
citrate	-ve
Gelatin hydrolysis	variable
Hemolysis	Variable(Alfa,Beta,Gama)
Growth in 6.5% NaCl	+ve
Bile .Esculin	+ve
Fermentation	
Glucose	+ve

Fructose	+ve
Maltose	+ve
Sucrose	+ve

Production of biofilm by *E.faecalis*

100% of E. faecalis have the capability to produce a biofilm was illustrated in table (3) the results revealed that all E.faecalis were biofilm former where all the isolates show that ability (100%).

Table 3: Production of biofilm in *E.faecalis*

Bacterial isolates NO	Biofilm production percentage			
	strong	moderate	weak	biofilm formation %
E.faecalis (7,20)	5(72%)	2(28%)	Zero	100%

Adherence of E. faecalis to oral epithelial cell.

The pathogenesis of bacterial infection started with adherence that facilitated by action of several adhesions located on the surface of bacteria, the results obtained from the current study shown that *E. faecalis* have the capability of adherence to oral epithelial cells.

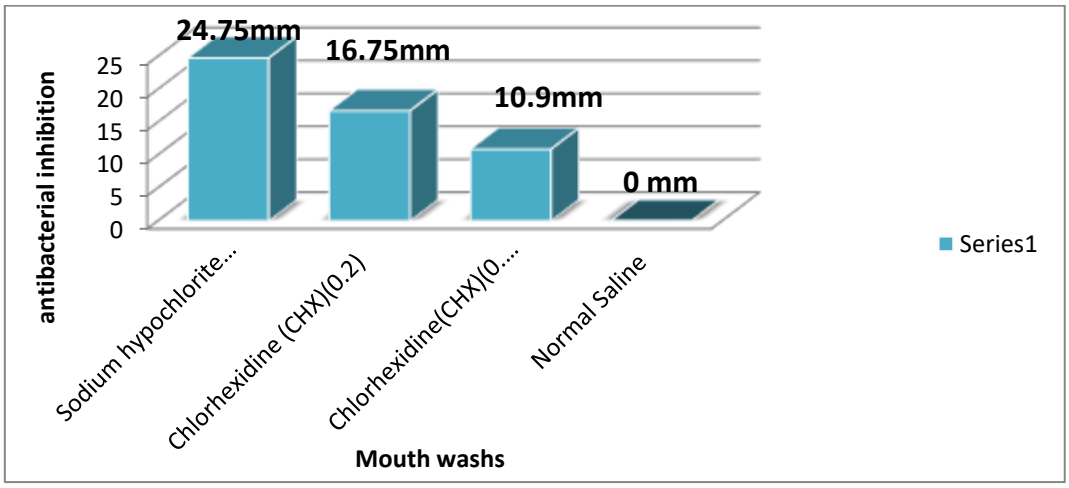

Figure 1: Effective of irrigation solutions on *E. faecalis* isolates

Antibacterial activity of irrigation solutions against *E.faecalis*

The results obtained from the preliminary analysis for effectiveness of antibacterial activity of irrigation solutions against *E.faecalis* in vitro are summarized in figure (1) the most supersizing aspect the data showed that NaOCl (5.25%) was associated with bigger inhibition zone (about 25 mm) compared to (11-17mm) with CHX group.

DISCUSSION

Unfortunately, *E.faecalis* is frequently found in infected root canal of teeth. Thus, this bacteria is isolated in cases of primary and secondary endodontic infections and it is established in endodontic infection and maintains a periradicular inflammation due to its virulence factors. So this microorganism alone has the capability to maintain root canal infection and periradicular lesion (19,20).

The results of the current study summarized that there was a correlation between the E.faecalis was isolated from endodontic infections and age. the present data was showed that patients aged (10-30) years represented about 32 % of the total samples and (47%) out of all the samples collected with positive results. Similarly, patients aged from (31-50) years in the rate of (45%), in contrast, the age range from (51-70) were associated with low rate (14%).

The current results matched with (21) who found that there was a positive correlation between age and *E.faecalis* isolated from endodontic infections. A possible explanation for this might be that *E.faecalis* is commonly detected in endodontic infections due to characteristic of this bacteria such as their virulence factors including protease, gelatinase, biofilm formation. In addition, it is considered as the microorganisms mainly responsible for persistent periradicular lesions even after root canal treatment. It can survive in the root canal as a single organism or as a major component of the flora (22).

Moreover, the findings of the current study are consistent with (23) who demonstrated that the aging process are associated with many alterations in the histological architecture of the dental pulp.

Consistently, (24) demonstrated that dentinal tubules of the young adult teeth were contained higher number of

bacteria, because ability of bacteria to deep penetration of dentinal tubules and cause root canal infections.

On the other hand, regarding to characteristic of *E.faecalis*, laboratory detection of *E.faecalis* isolated from endodontic infections may be confirmed by traditional method that including (biochemical test and microscopic examination, as well as phenotypic variants) which summarized in the current study. Seventy nine of the root canal samples shown growth when cultured but only one sample shown no growth, *although, E.faecalis* is easily incubated in ordinary non-selective media, similarly, growing under the conditions created for streptococci but to increase chance of finding it was cultured on Mitis Salivarius agar that the bacterial isolates have ability to grow on this media at anaerobic condition which shown blue, black, shiny and slightly raised colony. The bacterial isolates observed under the microscope are characterized by ovoid, single, pairs, or short chains and Gram staining appeared that *E. faecalis* isolates, Gram-positive cocci (18, 25).

In addition, most negative tests appeared in catalase test, indole test and Simon citrate test, while positive tests are demonstrated in bile esculin test that hydrolysis of esculin and growth in 6.5% NaCl due to the presence of bile salts, and sugar fermentation. Also variable results that were appeared on blood agars it is non-hemolytic or displays an α-hemolysis. In contrast, other studies recorded presence of a clear halo (β-hemolysis) around the colonies after incubation for 24 hours at 37°C was considered as positive for haemolysin (26, 27). The essential test that was used to presumptively identify *E.faecalis* was bile esculin test. The mechanisms of detection depend on the hydrolysis of esculin in the media into glucose and esculetin that.. As a result, the darke color may be indicate to positive bacterial isolates.

In this study *E. faecalis have capability to ferment multiple sugars such as (glucose, fructose, maltose, sucrose)* which used a homolactic fermentative pathway to produce lactic acid, Besides, to identify the bacteria (28).

Virulence factor

On the other hand , the results of virulence factors of E.faecalis will discuss, firstly started with biofilm production .The current study shown that most *E.faecalis* isolates were biofilm producer, and have ability to produce moderate and high biofilm production by *E.faecalis* at rate (100%),while there are no bacterial isolates classified as non-biofilm producer (28%,72%, 0%) respectively (29, 30).

This finding is in agreement with (30, 31) findings which showed the ability of *E. faecalis* isolates to form biofilms, it might be related to presence of the virulence determinants *efaA, esp, asa1*, *gelE* (32).

Secondly , the results of this study illustrated that the ability of *E.faecalis to* adherence to oral epithelial cell and tooth surface and it was consider as the initial step in infection .

Recently, the results of this study support the idea of (33) who revealed an increased interest in adhesion of *E. faecalis* is a normal inhabitant of the oral cavity and it is associated with different forms of peri-radicular diseases including primary endodontic infections and persistent infections. The visualization and quantification of adherent bacteria are still one of the challenges in dentistry.

the present findings is in agreement with (34,35)showed that *E. faecalis* have the ability of adhesion and penetration of root cementum provide a long-term nidus for subsequent infection, therefore, causing the persistent infection or reinfection that lead to endodontic infection and ability of bacterial adhesion to dentinal tubule walls is a logical early step in the process.

Furthermore, the result of present study consent with prior study (36) have noted that the time have important role in bacterial adhesion, abundance of E.faecalis was decreased when the duration of adhesion was increased.

Thirdly, the results of current data was appeared that the capability of *E.faecalis* to produce gelatinase enzyme, which detected in 90% (n=18) of most isolates by phenotypic method.

This finding is in agreement with (30) who showed that gelatinase activity was detected in almost all isolates of *E.faecalis*. There are several possible explanations for this result, firstly *E. faecalis* possesses several virulence factors that have special role in endodontic infections. Moreover, gelatinase is one of the virulence factor that were most extensively studied may and be associated with the survival of *E. faecalis* in root canals, in addition, it is one of important virulence factors responsible for initiation of root canal infection (37, 38).

In contrast to these findings, (39,37)evaluated the capability of *E. faecalis* strains to survive with and without gelatinase producing ability and showed that the production of gelatinase as putative virulence determinants were not always expressed by *E. faecalis* isolates in association with dental diseases, since only 37.5% gelatinase activity in vitro.

Last trait was ability of *E.faecalis* to produce Protease. The data of present study was revealed that over half of the isolates (60%,n=13) of *E.faecalis* have the ability to produce protease. In consistency with (41) who reported that commensal strains of *E. faecalis* may produce the protease.

This finding supports previous research which links protease production with *E.faecalis*. Thus *E. faecalis* secretes protease which is playing an important role in cleaving peptide bonds and helps the binding of *E. faecalis* to dentin, can result in invasion of bacteria the dentinal tubules and cause root canal infections (42).

Additionally, literature of (43) demonstrated findings about *E. faecalis* showing that it is able to co-colonize with other organisms in root canals and may depend on its protease production. High producers of proteases would suppress the growth of other, but not all, organisms in a biofilm consortium, whereas low producers would be able to coexist well with other species. As proteases can also cause tissue damage and stimulate immune responses, high producers may also represent more virulent *E. faecalis* strains.

In contrast with the results of present study, a recent study by (44) reports that *E. faecalis* proteolytic activity are represented by GelE and SprE., this protective mechanism is

primarily composed of GelE and another unidentified protease.

Finally, the current study was discussed the effect of irrigation solutions against *E.faecalis.* Undoubtedly, NaOCl is the most frequently recommended and a commonly used endodontic irrigant. Its advantages are two-fold; organic dissolution and antimicrobial effect. Because of that using of NaOCl decrease the microbial number so that it was consider as drug of chose for treatment endodontic infections (45). On the other hand, CHX is a cationic bisbiguanide antiseptic. Its advantages are based on a broad spectrum of activity (46).

The result of this investigation show that sodium hypochlorite(NaOCl)(5.25%) may present better antimicrobial activity with average (24.75mm) than Chlorhexidine (CHX) (0.2%) and CHX(0.12%) shown average(16.75mm,10.9mm) respectively.

This finding is in agreement with (47, 48) findings which showed 5.25% NaOCl is more effective than 2%chlorhexidin and must be still considered irrigate of choice. Similarly, (49) found that only NaOCl have the ability to eliminate biofilm after (1-3 minutes) of direct contact

Conversely, (50) showed that CHX is the irrigant of choice in endodontic treatments because it has a broad antimicrobial spectrum that also includes the anaerobic bacteria associated with endodontic treatment failure. CHX substantial effectiveness and low tissue toxicity further justify its use as an intracanal antimicrobial irrigant. Studies have shown that even though CHX is an effective disinfectant, it was not a curative drug (51). Additionally, *the present data was in contrast with* (52) who reported that CHX could be recommended to be incorporated in dressings and obturating pastes for teeth with periradicular pathosis to effectively kill most of the bacteria (including *E.faecalis*) within dentinal tubules of teeth.

CONCLUSION

This study has shown that there is a relationship between *E.faecalis* isolated from root canal infected cases and age, About 90% of *E.faecalis* has ability for production of gelatinase, 60% has ability to protease production and most isolates have ability of adherence to oral epithelial cell and 100% biofilm production. The results of this study showed that the irrigation solutions (NaOCl) (5.25%) have higher inhibition effect against *E.faecalis* than (CHX) (0.2%) and (CHX) (0.12%).

ACKNOWLEDGMENT

The author would like to thank the staff of conservative, operative and microbiology department in college of dentistry, university of Babylon Iraq.

REFERENCES

1. Rams, T. E., Degener, J. E., & van Winkelhoff, A. J. (2013). Prevalence of β-lactamase-producing bacteria in human periodontitis. Journal of periodontal research, 48(4), 493-499.
2. Rodríguez-Niklitschek, C. (2015). Clinical implications of Enterococcus faecalis microbial contamination in root canals of devitalized teeth: Literature review. Revista Odontológica Mexicana, 19(3), 181-186.
3. Zoletti, G. O., Pereira, E. M., Schuenck, R. P., Teixeira, L. M., Siqueira Jr, J. F., & dos Santos, K. R. N. (2011). Characterization of virulence factors and clonal diversity of Enterococcus faecalis isolates from treated dental root canals. Research in microbiology, 162(2), 151-158.
4. Flanagan, D. (2017). Enterococcus faecalis and dental implants. Journal of Oral Implantology, 43(1), 8-11.
5. Gajan, E.B.,Aghazadeh, M., Abashov, R.,Milani, A. S., & Moosavi, Z.(2009). Microbial flora of root canals of pulpally-infected teeth: Enterococcus faecalis a prevalent species. *Journal of dental research, dental clinics, dental prospects*, *3*(1), 24.
6. Ricucci, D., & Siqueira Jr, J. F. (2010). Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. *Journal of endodontics*, *36*(8), 1277-1288.
7. Hargreaves KM, Cohen S. Cohen's pathway of the pulp. 10th ed.. India: Elsevier; 2011. p. 529-55.
8. Rams, T. E., Degener, J. E., & van Winkelhoff, A. J. (2013). Prevalence of β-lactamase-producing bacteria in human periodontitis. Journal of periodontal research, 48(4), 493-499.
9. Anderson, A. C., Al-Ahmad, A., Elamin, F., Jonas, D., Mirghani, Y., Schilhabel, M., & Rehman, A. (2013). Comparison of the bacterial composition and structure in symptomatic and asymptomatic endodontic infections associated with root-filled teeth using pyrosequencing. PloS one, 8(12), e84960.
10. Al-Ahmad, A., Ameen, H., Pelz, K., Karygianni, L., Wittmer, A., Anderson, A. C., & Hellwig, E. (2014). Antibiotic resistance and capacity for biofilm formation of different bacteria isolated from endodontic infections associated with root-filled teeth. Journal of Endodontics, 40(2), 223-230.
11. Souto, R., & Colombo, A. P. V. (2008). Prevalence of Enterococcus faecalis in subgingival biofilm and saliva of subjects with chronic periodontal infection. Archives of oral biology, 53(2), 155-160.
12. Fouad, A. F., & Burleson, J. (2003). The effect of diabetes mellitus on endodontic treatment outcome: data from an electronic patient record. The Journal of the American Dental Association, 134(1), 43-51.
13. Rôças, I. N., Siqueira Jr, J. F., & Santos, K. R. (2004). Association of Enterococcus faecalis with different forms of periradicular diseases. Journal of endodontics, 30(5), 315-320.
14. Hancock III, H. H., Sigurdsson, A., Trope, M., & Moiseiwitsch, J. (2001). Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology, 91(5), 579-586.
15. Sunde, P. T., Olsen, I., Debelian, G. J., & Tronstad, L. (2002). Microbiota of periapical lesions refractory to

endodontic therapy. Journal of endodontics, 28(4), 304-310.

16. Stuart, C. H., Schwartz, S. A., Beeson, T. J., & Owatz, C. B. (2006). Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. Journal of endodontics, 32(2), 93-98.
17. Razavian, H., Barekatain, B., Shadmehr, E., Khatami, M., Bagheri, F., & Heidari, F. (2014). Bacterial leakage in root canals filled with resin-based and mineral trioxide aggregate-based sealers. *Dental research journal*, *11*(5), 599.
18. Endo, M. S., Signoretti, F. G., Kitayama, V. S., Marinho, A. C., Martinho, F. C., & Gomes, B. P. F. A. (2014). Culture and molecular detection of Enterococcus faecalis from patients with failure endodontic treatment and antimicrobial susceptibility of clinical isolates. Braz Dent Sci, 17(3), 83-91.
19. Farac RV, Morgental RD, Lima RK, et al. Pulp sensibility test in elderly patients. Gerodontology 2012;29:135–9.
20. Rodríguez-Niklitschek, C. (2015). Clinical implications of Enterococcus faecalis microbial contamination in root canals of devitalized teeth: Literature review. Revista Odontológica Mexicana, 19(3), 181-186.
21. Pourhajibagher, M., Ghorbanzadeh, R., & Bahador, A. (2017). Culture-dependent approaches to explore the prevalence of root canal pathogens from endodontic infections. Brazilian oral research, 31.
22. Mallick, R., Mohanty, S., Behera, S., Sarangi, P., Nanda, S., & Satapathy, S. K. (2014). Enterococcus faecalis: A resistant microbe in endodontics. International Journal of Contemporary Dental & Medical Reviews, 2014.
23. Aslantas, E. E., Buzoglu, H. D., Karapinar, S. P., Cehreli, Z. C., Muftuoglu, S., Atilla, P., & Aksoy, Y. (2016). Age-related changes in the alkaline phosphatase activity of healthy and inflamed human dental pulp. Journal of endodontics, 42(1), 131-134.
24. Kakoli, P., Nandakumar, R., Romberg, E., Arola, D., & Fouad, A. F. (2009). The effect of age on bacterial penetration of radicular dentin. Journal of endodontics, 35(1), 78-81.
25. Soheili, S., Ghafourian, S., Sekawi, Z., Neela, V., Sadeghifard, N., Ramli, R., & Hamat, R. A. (2014). Wide distribution of virulence genes among Enterococcus faecium and Enterococcus faecalis clinical isolates. The Scientific World Journal, 2014.
26. Sedgley, C. M., Lennan, S. L., & Clewell, D. B. (2004). Prevalence, phenotype and genotype of oral enterococci. Oral microbiology and immunology, 19(2), 95-101.
27. Zoletti, G. O., Pereira, E. M., Schuenck, R. P., Teixeira, L. M., Siqueira Jr, J. F., & dos Santos, K. R. N. (2011). Characterization of virulence factors and clonal diversity of Enterococcus faecalis isolates from treated dental root canals. Research in microbiology, 162(2), 151-158.
28. Subramanian, M. R., Talluri, S., & Christopher, L. P. (2015). Production of lactic acid using a new homofermentative E nterococcus faecalis isolate. Microbial biotechnology, 8(2), 221-229.
29. Ran, S. J., Jiang, W., Zhu, C. L., & Liang, J. P. (2015). Exploration of the mechanisms of biofilm formation by Enterococcus faecalis in glucose starvation environments. Australian dental journal, 60(2), 143-153.
30. Aghdam, M. A., Barhaghi, M. S., Aghazadeh, M., Jafari, F., Hagh, M. B., Haghdoost, M., .. & Kafil, H. S. (2017). Virulence genes in biofilm producer Enterococcus faecalis isolates from root canal infections. Cell Mol Biol (Noisy le Grand), 63(5).
31. Zheng, J. X., Wu, Y., Lin, Z. W., Pu, Z. Y., Yao, W. M., Chen, Z., ... & Yu, Z. J. (2017). Characteristics of and virulence factors associated with biofilm formation in clinical Enterococcus faecalis isolates in China. *Frontiers in microbiology*, *8*, 2338.
32. Hashem, Y. A., Amin, H. M., Essam, T. M., Yassin, A. S., & Aziz, R. K. (2017). Biofilm formation in enterococci: genotype-phenotype correlations and inhibition by vancomycin. *Scientific reports*, *7*(1), 5733.
33. Nair, V. S., Nayak, M., Ramya, M. K., Sivadas, G., Ganesh, C., Devi, S. L., & Vedam, V. (2017). Detection of adherence of Enterococcus faecalis in infected dentin of extracted human teeth using confocal laser scanning microscope: An In vitro Study. Journal of pharmacy & bioallied sciences, 9(Suppl 1), S41.
34. Halkai, R. S., Hegde, M. N., & Halkai, K. R. (2016). Evaluation of Enterococcus faecalis adhesion, penetration, and method to prevent the penetration of Enterococcus faecalis into root cementum: Confocal laser scanning microscope and scanning electron microscope analysis. Journal of conservative dentistry: JCD, 19(6), 541.
35. Halkai, R., Hegde, M. N., & Halkai, K. (2012). Root cementum invasion and adhesion by Enterococcus faecalis confocal analysis. NUJHS, 2, 44-9.
36. Djimeli, C. L., Arfao, A. T., Rossi, V., Nsulem, N., Raspal, V., Bricheux, G., ... & Sime-Ngando, T. (2016). Impact of two disinfectants on detachment of Enterococcus faecalis from polythene in aquatic microcosm. Research in Biotechnology, 7.
37. Sedgley, C. M. (2007). The influence of root canal sealer on extended intracanal survival of Enterococcus faecalis with and without gelatinase production ability in obturated root canals. Journal of endodontics, 33(5), 561-566.
38. Bhardwaj, B. S. (2013). Role of Enterococci faecalis in failure of Endodontic treatment. Int J Curr Microbiol App Sci, 2(8), 272-277.
39. Salah, R., Dar-Odeh, N., Hammad, O. A., & Shehabi, A. A. (2008). Prevalence of putative virulence factors and antimicrobial susceptibility of Enterococcus faecalis isolates from patients with dental Diseases. BMC Oral Health, 8(1), 17.
40. Steck, N., Hoffmann, M., Sava, I. G., Kim, S. C., Hahne, H., Tonkonogy, S. L., & Vogelmann, R. (2011). Enterococcus faecalis metalloprotease compromises epithelial barrier and contributes to

intestinal inflammation. Gastroenterology, 141(3), 959-971.

41. Steck, N., Hoffmann, M., Sava, I. G., Kim, S. C., Hahne, H., Tonkonogy, S. L., & Vogelmann, R. (2011). Enterococcus faecalis metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. Gastroenterology, 141(3), 959-971.
42. Provenzano, J. C., Siqueira Jr, J. F., Rôças, I. N., Domingues, R. R., Leme, A. F. P., & Silva, M. R. (2013). Metaproteome analysis of endodontic infections in association with different clinical conditions. PLoS One, 8(10), e76108.
43. Chávez de Paz, L. E., Davies, J. R., Bergenholtz, G., & Svensäter, G. (2015). Strains of Enterococcus faecalis differ in their ability to coexist in biofilms with other root canal bacteria. International endodontic journal, 48(10), 916-925.
44. Nešuta, O., Buděšínský, M., Hadravová, R., Monincová, L., Humpoličková, J., & Čeřovský, V. (2017). How proteases from Enterococcus faecalis contribute to its resistance to short α-helical antimicrobial peptides. Pathogens and disease, 75(7), ftx091.
45. Vijaykumar, S., GunaShekhar, M., & Himagiri, S. (2010). In vitro effectiveness of different endodontic irrigants on the reduction of Enterococcus faecalis in root canals.
46. Bonsor, S. J., Nichol, R., Reid, T. M. S., & Pearson, G. J. (2006). Microbiological evaluation of photo-activated disinfection in endodontics (an in vivo study). *British dental journal*, *200*(6), 337.
47. Estrela, C., Silva, J. A., Alencar, A. H. G. D., Leles, C. R., & Decurcio, D. A. (2008). Efficacy of sodium hypochlorite and chlorhexidine against Enterococcus faecalis: a systematic review. *Journal of Applied oral science*, *16*(6), 364-368.
48. Agrawal, V., Rao, M. R., Dhingra, K., Gopal, V. R., Mohapatra, A., & Mohapatra, A. (2013). An in vitro comparison of antimicrobial effcacy of three root canal irrigants-BioPure MTAD, 2% chlorhexidine gluconate and 5.25%sodium hypochlorite as a final rinse against E. faecalis. *J Contemp Dent Pract*, *14*(5), 842-7.
49. Nascimento, C. A., Tanomaru-Filho, M., Faria-Junior, N. B. D., Faria, G., & Guerreiro-Tanomaru, J. M. (2014). Antimicrobial activity of root canal irrigants associated with cetrimide against biofilm and planktonic Enterococcus faecalis. *The journal of contemporary dental practice*, *15*(5), 603-607.
50. Ahmetoglu, F., Keles, A., Yalcin, M., & Simsek, N. (2014). Effectiveness of different irrigation systems on smear layer removal: A scanning electron microscopic study. *European journal of dentistry*, *8*(1), 53.
51. Piovesani, J. F., Semenoff-Segundo, A., Pedro, F., Borges, A. H., Neves, A. N. P., Mamede Neto, L., & Semenoff, T. A. D. V. (2012). antibacterial capacity of different intracanal medications on enterococcus faecalis. *Dental Press Endod*, *2*(2), 53-8.
52. Verma, R., Sharma, D. S., & Pathak, A. K. (2015). Antibacterial Efficacy of Pastes Against E Faecalis in Primary Root Dentin: A Confocal Microscope Study. *Journal of Clinical Pediatric Dentistry*, *39*(3), 247-254.