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# The effect of gelatinase production of *Enterococcus faecalis* on adhesion to dentin after irrigation with various endodontic irrigants

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**ABSTRACT**

**Objectives:** The aim of this study was to evaluate whether the gelatinase production ability of *Enterococcus faecalis* provides any advantage on adhesion of this bacterium to dentin treated with various irrigants and their combinations.

**Materials and methods:** Standardized dentin discs were randomly divided into five groups ($n = 20$): group 1: 2.5% sodium hypochlorite (NaOCl), group 2: 2% chlorhexidine (CHX), group 3: NaOCl + Saline + CHX, group 4: NaOCl + EDTA + NaOCl, group 5: QMix. After incubation of dentin discs with irrigants, each group was divided into two subgroups ($n = 10$) according to the bacterial strains used; a gelatinase-producing and a gelatinase-deficient strain of *E. faecalis.* After incubation of the discs with the bacterial suspensions aerobically for 48 h, XTT assay was conducted for bacterial adherence evaluation. Data were statistically analyzed by ANOVA and Tukey's HSD tests ($p = .05$).

**Results:** Gelatinase-producing *E. faecalis* adhered to dentin was significantly more than gelatinase-deficient *E. faecalis* in all test groups ($p < .05$). Adherence to CHX-treated dentin was lower than to the surfaces treated with other irrigants, alone or in combination ($p < .05$). These differences were significant except for comparisons with QMix for gelatinase-producing bacteria ($p < .05$).

**Conclusions:** Gelatinase production of *E. faecalis* may be an important factor for bacterial adhesion. The addition of CHX to the irrigation regimen resulted in fewer adhered bacteria to dentin. QMix was not as effective as CHX in terms of bacterial adhesion prevention.

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## Introduction

Bacteria are commonly found within dentinal tubules of clinically infected root canals. Among these bacteria, *Enterococcus faecalis* is often recovered from treated endodontic cases with persistent lesions, whereas it is more rarely detected in necrotic pulp tissues.[1,2] It has been proposed that *E. faecalis* are transient bacteria of the oral cavity, possibly coming from fermented food products, but if *E. faecalis* finds a suitable habitat allowing their growth and survival, such as unsealed necrotic or filled canals, they might enter and survive and multiply there.[3]

Bacterial adhesion to dentin and invasion of dentinal tubules is considered important in the establishment of endodontic infections.[4] The primary bacterial adhesion depends on specific adhesion characteristics of bacteria as well as surface characteristics of dentin.[5] Gelatinase production, a virulence factor of *E. faecalis*, may contribute to the survival of bacteria in filled root canals.[6] For example, it has been recently reported that gelatinase expression is related to the enhancement of biofilm formation *in vitro*.[7] However, it is not yet clear whether gelatinase production plays an important role in the adherence of *E. faecalis* to dentin.

Irrigation is a crucial endodontic procedure for the elimination of microorganisms from the root canal system.[8] Sodium hypochlorite (NaOCl) is one of the most frequently used irrigant because of its ability to destroy a broad spectrum of microorganisms and dissolve organic materials.[9] The alternating use of ethylenediaminetetraacetic acid (EDTA) and NaOCl has long been proved effective in removing smear layer.[10] Chlorhexidine gluconate (CHX) has been suggested as an endodontic irrigant due to its antimicrobial effects, and it has lower cytotoxicity but greater substantivity than NaOCl.[11]

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Recently, another endodontic irrigant containing EDTA, CHX and a detergent has been introduced as QMix (Dentsply Tulsa Dental, Tulsa, OK).

After a root canal instrumentation or filling, bacteria can re-invade the root canal system via coronal leakage.[12] Adhesion of bacteria to the root canal dentin is prerequisite for the development of the endodontic infections.[13] Most microbiological studies in endodontics have searched the antimicrobial capacity of endodontic irrigants, but very few studies have focused on the efficacy of different irrigation regimens on the adherence of *E. faecalis* to dentin.[14–17]

Therefore, the aim of this *in vitro* study was to evaluate the effects of different endodontic irrigants as a final rinse on the adherence of the gelatinase-producing and the gelatinase-deficient *E. faecalis* strains to dentin using the XTT ((2,3)-bis(2-methoxy-4-nitro-5- sulfophenyl)-5-[(phenylamino)-carbonyl]-2*H*-tetrazolium hydroxide) colorimetric assay.

## Materials and methods

This research was conducted according to strict compliances outlined by World Medical Association Declaration of Helsinki. The teeth used in this study were extracted for medical reasons, and all patients gave informed consent before sample collection. The bacterial adhesion method was adapted from Sen et al. [18].

### Dentin discs preparation

Non-carious third molars ($n = 120$) were used in this study. The surrounding enamel was removed with a high-speed industrial cutting instrument under copious water irrigation to get standardized 5 mm width anatomical crowns. Dentin discs with an approximate thickness of 1.2 mm were cut just inside the deepest part of the occlusal dentinoenamel junction by using a rotary diamond saw (ISOMET, Buehler, Lake Bluff, NY), rendering discs that were ground on both sides. Only one dentin disc was prepared from each tooth. Complete removal of enamel was verified under the stereo-microscope (SZTP; Olympus Optical Co, Tokyo, Japan) at 15× magnification. The discs were ground with wet sandpaper (#400 to #1200 grit) to create smooth surfaces and to reduce the thickness of the discs to 1.0 mm, as measured with a micrometer (Miltex, Tuttlingen, Germany). This procedure created smear layer on all surfaces of the discs. Standardization of each sample was confirmed by calculating the total surface area using a software program (Leica Stereo Explorer, Leica Microsystems Ltd, Heerbrugg, Switzerland).

### Microorganisms and culture conditions

Two clinical strains of *E. faecalis* [the gelatinase-producing *E. faecalis* (OG1RF) and the gelatinase-deficient *E. faecalis* (TX5128)] were obtained (Selcuk University, Faculty of Medicine, Microbiology Department) and used. The strains were grown overnight at 37 °C in aerobic conditions in tryptone soy broth (TSB; BioMerieux, Charbonnieresles-Bains, France). A loopful of *E. faecalis* was incubated in 5 mL of TSB at 37 °C overnight. The optical density of bacterial suspension was standardized with a spectrophotometer at 600 nm wavelength to 600.

### Treatment groups of dentin discs with different irrigation regimens

The prepared dentin discs were transferred individually into the prenumbered Eppendorf tubes containing phosphate-buffered saline (PBS, pH = 7.2, Sigma-Aldrich, St. Louis, MO) and were autoclaved at 121 °C for 20 min. The samples were randomly divided into five test groups ($n = 20$), and a negative control group ($n = 20$), as shown below:

Group 1: 5 ml of 2.5% Sodium hypochlorite (NaOCl) (Caglayan Kimya, Konya, Turkey) (15 min)

Group 2: 5 ml of 2% Chlorhexidine (CHX) (Klorhex, Drogsan, Turkey) (15 min)

Group 3: 5 ml of 2.5%NaOCl + 5 ml Saline (I.E Uragay, İstanbul, Turkey) + 5 ml 2% CHX (5 min per each irrigant)

Group 4: 5 ml of 2.5% NaOCl + 5 ml 17% EDTA (Merck KGaA, Darmstadt, Germany) + 5 ml 2.5% NaOCl (5 min per each irrigant)

Group 5: 5 ml of QMix (Dentsply Tulsa Dental, Tulsa, OK) according to the manufacturer's recommendations, dentin discs were irrigated with 5 ml 5.25% NaOCl for 5 min, 5 ml sterile saline solution for 5 min and QMix (90 sec), respectively. The concentration of NaOCl and irrigation time with QMix was the strict instructions of QMix irrigation protocol.

Negative control group: Sterilized dentin discs incubated with sterile TSB solution without any bacterial infection.

After treatment of dentin discs with different irrigation regimens, discs were rinsed with 5 ml of distilled water. Each group was then divided into two subgroups ($n = 10$) according to the bacterial strains used (*E. faecalis* [OG1RF] and *E. faecalis* [TX5128]). Treated dentin discs were placed in sterile 24-well

tissue culture plates, with 1 disc in each well. Bacterial cells were standardized to $1 \times 10^8$ cells/mL in TSB. Aliquots of 1 ml of bacterial suspensions were transferred into each well and incubated aerobically for 48 h at 37 °C without shaking. After 24 h, bacterial suspension was refreshed with the addition of 1 ml of fresh media.

### *XTT reduction assay*

A quantitative measurement of bacterial adhesion was determined by using an XTT-reduction assay, adapted according to the manufacturer's instructions and as described previously.[18,19] XTT test is based on the metabolically active cells to reduce 2,3-bis(2-methoxy-4 -nitro-5-sulphophenyl)- 2H-tetrazolium-5-carboxanilide (XTT) molecules to a soluble salt of formazan, detectable by its absorbance at 480 nm.

XTT (SERVA Electrophoresis, Heidelberg, Germany) was dissolved in PBS to a final concentration of 1 mg $mL^{-1}$ and filter-sterilized. Phenazine methosulphate (PMS, Sigma-Aldrich, St. Louis, MO) solution was prepared and filtered. XTT solution was thawed and mixed with PMS solution at a volume ratio of 20:1 before each assay. After 48 h of bacterial incubation, the dentin discs were washed twice with 600 µl PBS for removal of nonadherent cells. The samples were then gently transferred to 96-well tissue culture plates containing 158 µl PBS, 40 µl XTT and 2 µl PMS per well.[19] After incubation in the dark at 37 °C for 3 h, 100 µl of the solution without dentin discs was transferred to new sterile 96-well plates. The spectrophotometer was calibrated with 1 mL of pure PBS solution containing the same concentration of XTT and PMS without *E. faecalis* cells and dentin discs.

A colorimetric change in the XTT reduction assay, a direct correlation of the metabolic activity of the bacteria, was then analyzed at 492 nm using a spectrophotometer (µ Quant ELISA Reader, Bio-Tek Instruments, Winooski, VT). The optical density (OD) values, measured by XTT reduction assay, demonstrated the adhered *E. faecalis* cells on dentin. Data were analyzed using one-way analysis of variance and the *post hoc* Tukey tests. The level of statistical significance was set at $p = .05$.

## Results

Gelatinase-producing *E. faecalis* adhered to dentin significantly more than gelatinase-deficient *E. faecalis* in all test groups ($p < .05$). The negative control group showed no bacterial growth on dentin surfaces (OD value = 0). Table 1 shows the OD values of adherent gelatinase-producing and gelatinase-deficient *E. faecalis* strains to dentin discs treated with different irrigation regimens, respectively.

Dentin discs treated with the NaOCl group showed the highest OD values of adhered gelatinase-producing *E. faecalis* and gelatinase-deficient *E. faecalis* ($p < .05$). However, the adhesion of gelatinase-producing *E. faecalis* was significantly inhibited by the CHX group and the NaOCl + Saline + CHX group ($p < .05$). The QMix group could not reduce the OD values of gelatinase-deficient *E. faecalis* when compared with the CHX group and the NaOCl + Saline + CHX group ($p < .05$). No statistically significant difference was determined between the QMix group and the NaOCl + EDTA + NaOCl groups ($p > .05$).

## Discussion

In the present study, XTT assay was used to evaluate the number of *E. faecalis* cells adhered to dentin. XTT is a colorimetric microbiological tool used for evaluation of the metabolic activity of adherent aerobic bacteria and a measure of cell viability. This approach depends on the ability of metabolically active cells to convert the yellow water-soluble XTT into orange-colored soluble compounds of formazan, measured by their absorbance at 480 nm with a micro plate UV/Vis spectrophotometer. The XTT assay has the following important advantages over the use of microbiologic,

**Table 1.** The mean ± standard deviation of OD values of gelatinase-producing *E. faecalis* and gelatinase-deficient *E. faecalis* adhering to dentin.

| Test groups | OD values for gelatinase-producing *E. faecalis* | OD values for gelatinase-deficient *E. faecalis* |
|---|---|---|
| 2.5% NaOCl | $0.319 \pm 0.094^{c*}$ | $0.144 \pm 0.024^{c*}$ |
| 2% CHX | $0.021 \pm 0.010^{a}$ | $0.016 \pm 0.010^{a}$ |
| 2.5% NaOCl + Saline +2% CHX | $0.020 \pm 0.010^{a}$ | $0.014 \pm 0.09^{a}$ |
| 2.5% NaOCl + EDTA +2.5% NaOCl | $0.197 \pm 0.048^{b}$ | $0.111 \pm 0.028^{bc}$ |
| QMix | $0.129 \pm 0.047^{ab}$ | $0.084 \pm 0.013^{b}$ |

*The letters represent the statistical differences between irrigation solution test groups in each column on an individual basis. Groups identified by different superscript letters are significantly different ($p < .05$).

histological, and light and electron microscopic techniques:

1. Allows a fast quantitative measurement.
2. Determines the number of only living cells.
3. The possibility of working with whole biofilm, without destroying it.
4. Allows the counting of adherent cells on dentin, which is not possible with light microscopy because the dental tissues are not transparent [18–22].

No deactivating agent was used to reduce the carry-over effect of the disinfectant solutions, due to the lack of a universal neutralizing agent suitable for all the irrigants that were tested. Furthermore, the clinical use of irrigants does not necessitate neutralizing the irrigants, and some of them contain components that might show an antimicrobial effect on the biofilms, thereby masking the results [23]. Therefore, rinsing dentin discs with distilled water was preferred, following irrigation protocols that Sum et al. [16] performed in their bacterial adhesion study.

Gelatinase production is one of the virulence factors that may be associated with the survival of *E. faecalis* in filled root canals.[6,24] Gelatinases are extracellular zinc-containing endopeptidases that can hydrolyze gelatin, collagen, fibrin and other peptides.[25] In terms of virulence, a large proportion of *E. faecalis* that can produce gelatinases are frequently isolated from hospitalized patients.[26] In addition, increased lethality of a gelatinase-producing *E. faecalis* strain was reported compared to the isogenic strain that is deficient in gelatinase production.[27] From an endodontic viewpoint, it has been reported that the expression of gelatinase was higher in the biofilm-positive strains from cases of apical radiolucency.[7] The results of the present study may also be taken to suggest that gelatinase production could facilitate the adhesion of *E. faecalis* to dentin.

Although the role of gelatinase in enhancing bacterial dentin adhesion is not known in detail, Kristich et al. [24] reported some possible models. Gelatinase might have a role in the production of an extracellular signaling peptide molecule, thereby serving as a signal for biofilm formation. In an alternative model, gelatinase might proteolytically activate another surface protein that participates in the secretion of extracellular polymeric matrix material for biofilm formation.[24] Furthermore, gelatinase could produce peptides and amino acids that could be an important source for bacterial growth.[28] In addition, gelatinase may be able to significantly increase the bacterial cell surface hydrophobicity, thus enhancing attachment to a substratum.[29,30] Another possible explanation could be that the collagen degradation products such as collagen, fibrin or gelatin may be used by gelatinase-producing *E. faecalis* strain as nutrients.[30]

The alteration in dentin collagen caused by endodontic irrigants might be one of the reasons for apparently different *E. faecalis* adhesion values. In the present study, dentin discs irrigated with NaOCl had the highest values for bacterial adhesion on dentin. This could be caused by the fact that NaOCl can cause collagen denaturation[16], thereby increasing *E. faecalis* adhesion to dentin.[4,31,32] Another reason for the increased adhesion may be due to slightly alkaline-altered pH of collagen that encourages *E. faecalis* adhesion.[5,16]

CHX treatment with its sole use or in combination with NaOCl resulted in prevention of bacterial adhesion. The use of NaOCl that can remove exposed collagen fibrils [8] and subsequent CHX irrigation (NaOCl + Saline + CHX group) also significantly reduced the bacterial adhesion in this study. CHX can prevent microbial adhesion to dentinal surface for longer periods due to its substantivity property.[11,15] Therefore, it is not surprising that minimal bacterial adhesion was obtained when CHX was integrated into the irrigation regimen. Only short-term effect (48 h incubation periods) of the irrigant exposure to bacterial adhesion was studied but it is possible that the substantivity effect of CHX can eventually disappear. Therefore, further research with a longer incubation time should be performed in order to determine how long the antimicrobial effect of CHX will last on dentin surface.

The use of QMix with 5.25% NaOCl combination instead of its single use in the present study was based on the strict instructions of the manufacturer. Hence, we irrigated the specimens initially with 5.25% NaOCl for 5 min; sterile saline solution for 5 min and finally QMix solution for 90 s in this group. To our knowledge, this is the first study that evaluated the adhesion of *E. faecalis* to dentin treated with QMix. The present study showed that bacterial adhesion was higher in QMix group when compared with CHX groups. Therefore, one may speculate from the results of this study that CHX solution was more effective in preventing bacterial adhesion when compared to QMix. The amount of CHX in QMix solution or a possible inactivation of CHX by EDTA which is also present in QMix solution may be the reasons for unsuccessful bacterial adhesion prevention. Future studies should be

conducted to determine the anti-adhesive capacity of QMix on other microorganisms isolated from endodontic infections.

## Conclusions

Within the limitations of this *in vitro* study, it can be concluded that gelatinase production of *E. faecalis* may be an important factor for its adhesion to dentin. The addition of CHX to the irrigation regimen resulted in fewer bacteria recovered from the surface. QMix was not as effective as CHX solution regarding bacterial adhesion prevention.

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## Disclosure statement

The authors deny any conflicts of interest.

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