**Effect of DNase Treatment on Adhesion and Early Biofilm Formation of Enterococcus Faecalis**

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**ABSTRACT**

**Objective:** Extracellular DNA (eDNA) has been shown to be important for biofilm stability of the endodontic pathogen Enterococcus faecalis. In this study, we hypothesized that treatment with DNase prevents adhesion and disperses young *E. faecalis* biofilms in 96-well plates and root canals of extracted teeth.

**Methods:** *E. faecalis* eDNA in 96-well plates was visualized with TOTO-1®. The effect of DNase treatment was assessed in 96-well plates and in extracted single-rooted premolars (n=37) using a two-phase crossover design. *E. faecalis* was treated with DNase (50 Kunitz/mL) or heat-inactivated DNase for 1 h during adhesion or after 24 h of biofilm formation. In 96-well plates, adhering cells were quantified using confocal microscopy and digital image analysis. In root canals, the number of adhering cells was determined in dentine samples based on colony forming unit counts. Data from the 96-well plate were analyzed using one-tailed t-tests, and data from extracted teeth were analyzed using mixed-effect Poisson regressions.

**Results:** eDNA was present in wells colonized by *E. faecalis* after 1 h of adhesion and 24 h of biofilm formation; it was removed by DNase treatment, as evidenced by TOTO®-1 staining. DNase treatment reduced the area covered by cells in 96-well plates after 1 h (P<0.05), but not after 24 h (P=0.98). No significant differences in the number of adhering cells were observed in extracted teeth after 1 (P=0.14) and 24 h (P=0.98).

**Conclusion:** DNase treatment does not disperse endodontic *E. faecalis* biofilms. The sole use of DNase as an anti-biofilm agent in root canal treatments is not recommended.

**Keywords:** Adhesion, biofilm; DNase, *Enterococcus faecalis*, endodontic biofilms, extracellular DNA

**HIGHLIGHTS**

- *E. faecalis* releases extracellular DNA, which can be removed by DNase treatment, during bacterial attachment and early biofilm formation.
- DNase treatment reduces the amount of *E. faecalis* cells adhering to 96-well plates, but it does not significantly reduce the amount of bacteria adhering to root canals of extracted teeth.
- DNase treatment does not disperse 24-h-old biofilms of *E. faecalis* formed in 96-well plates or in root canals of extracted teeth and is, thus, unlikely to be a valuable adjunct to endodontic therapy.

**INTRODUCTION**

Bacterial biofilms tolerate high concentrations of biocides; this property is partly attributed to the protective matrix in which bacterial cells are embedded. Therefore, treatment of bacterial biofilms with matrix-degrading enzymes has become a field of increasing research interest in the past years. Extracellular DNA (eDNA) entered the list of potential targets in the matrix when Whitchurch et al. (1) first demonstrated its importance for the mechanical stability of young *Pseudomonas aeruginosa* biofilms. eDNA is present in biofilms formed by a wide range of different species, including *Bordetella pertussis*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, *Streptococcus mutans* and *Streptococcus pneumoniae* (2–7), where it plays a role in the initial attachment of bacterial cells to the substratum, but also serves as a scaffold for biofilm architecture (8). Enzymatic digestion of eDNA with DNase has been shown to effectively disperse biofilms, particularly during initial stages of biofilm growth (9). For treatment of cystic fibrosis, a lung disease caused by *P. aeruginosa* biofilms, treatment with DNase has become the standard therapeutic approach to suppress biofilm formation (10).

Biofilm formation of *Enterococcus faecalis*, frequently associated with failed root canal treatment, also depends on the production of eDNA. Bacterial DNA is released by the action of the secreted metalloprotease gelatinase (GeE) on the major autolysin (AtlA), but only in a subpopulation of...
cells (ca 15%) that do not, concomitantly, express serine pro-
tease (SprE) for preventing autolysis. The released chromo-
somal eDNA then facilitates initial attachment and microcolony
formation (11, 12).

In addition to this fratricidal mechanism of release, lysis-in-
dependent secretion of eDNA from metabolically active cells
during early stages of biofilm formation has been described
(13). As E. faecalis is highly prevalent in endodontic retreat-
tment cases (14), rinsing with DNase might serve as a therapeu-
tic adjunct, provided that it prevents bacterial adhesion to
the canal wall and/or disperses established biofilms of E. faecalis.

It has previously been shown that DNase treatment reduces
the amount of cells recovered from E. faecalis biofilms grown
on dentine blocks (15), but thus far, no data are available on
the effect of DNase treatment on adhering cells of E. faecalis
in root canals. This study hypothesized that DNase treatment
reduced adhesion and dispersed young biofilms of E. faecalis,
in 96-well plates and extracted teeth. In this study, we, there-
fore, aimed a) to determine the effect of DNase treatment on E.
faecalis in 96-well plates after 1 h of adhesion and after 24 h of
biofilm formation using a combination of confocal microscopy
digital image analysis and b) to determine the effect of
DNase treatment on the number of attached E. faecalis cells to
the root canal wall of extracted teeth after 1 h of adhesion and
24 h of biofilm formation.

MATERIALS AND METHODS

Bacterial cultures
E. faecalis DSM 20478T was cultivated on blood agar plates (SSI,
Copenhagen, Denmark) at 35°C under aerobic conditions and
grown in tryptic soy broth (TSB, Scharlab, Barcelona, Spain) for
18 h. Prior to experimental use, cells were washed with fresh
TSB and adjusted to an optical density of 0.7 (550 nm) for ad-
hesion experiments (2×10^7 cells/mL) and of 0.4 (550 nm) for
biofilm experiments (10^8 cells/mL).

Effect of DNase treatment on bacterial adhesion and
biofilm dispersal in 96-well plates
For adhesion experiments, E. faecalis suspensions were mixed
with equal volumes of DNase I (Sigma Aldrich, Brøndby, Den-
mark; final concentration of 50 Kunitz/mL, 5 mM MgCl2 in 0.9%
NaCl) or heat-inactivated DNase I (100°C, 30 min), injected in
96-well plates (Ibidi μ-plate uncoated, Planegg/Martinsried,
Germany) in triplicates, and incubated for 1 h at 35°C in jars con-
taining anaerobic gas generating sachets (Anaerogen; Thermo
Fisher Scientific, Copenhagen, Denmark). Thereafter, wells were
washed five times with 0.9% NaCl for removing non-adherent
cells. For biofilm experiments, E. faecalis was injected in 96-well
plates, incubated for 24 h at 35°C under anaerobic conditions,
and then washed five times with 0.9% NaCl. Triplicate biofilms
were thereafter incubated for 1 h at 35°C in anaerobic jars with
DNase I or heat-inactivated DNase I and washed five times with
0.9% NaCl to remove unbound cells. Bacterial cells were stained
with SYTO® 9 (Thermo Fisher Scientific, Copenhagen, Denmark;
3.3 μM in 0.9% NaCl) and imaged using a confocal laser scanning
microscopy (Zeiss LSM 510 META, Jena, Germany) equipped
with a 20×/0.8 NA objective (Plan-Apochromat, Zeiss, Jena,
Germany) and a 488-nm laser line. Detection was performed from
501 to 554 nm; three images (450×450 μm) per well were
acquired with the following settings: Pinhole 3 Airy units (opti-
cal slice, 117 μm), pixel dwell time 3.29 μs, line average 2, and
resolution 0.22 μm/pixel. Images were then exported to the
software daime (16), segmented with appropriate brightness
thresholds, and the area covered by bacteria was determined.
All experiments were performed in biological triplicates.

Visualization of eDNA
Cells of E. faecalis were allowed to adhere or form biofilms, as
described above. Non-adherent cells were removed by washing
five times with 0.9% NaCl. eDNA and dead (permeable) cells were
visualized by staining with the membrane-impermeant nucleic
acid stain TOTO-1® (2 μM; 15 min incubation time; Thermo Fisher
Scientific, Copenhagen, Denmark), whereas remaining cells
were stained with the membrane-permeant nucleic acid stain
SYTO® 60 (10 μM; incubation time, 15 min; Thermo Fisher Sci-
entific, Copenhagen, Denmark). Excitation was performed with
514- (TOTO-1®) and 633-nm (SYTO® 60) laser lines. Detection
was performed from 522 to 597 nm (TOTO-1®) and from 640 to
704 nm (SYTO® 60). Images were acquired using the 20×/0.8 NA
objective (450×450 μm image size; 1 Airy unit pinhole (optical
slice, 60 μm), pixel dwell time 1.6 μs, line average 2, and resolu-
tion 0.22 μm/pixel) or a 63×/1.2 NA water immersion objective
(C-Apochromat, Zeiss; 143×143 μm image size; 1 Airy unit pin-
hole (optical slice, 0.9 μm), pixel dwell time 1.6 μs, line average
2, and resolution 0.07 μm/pixel). Experiments were performed
in biological duplicates.

Selection and preparation of teeth
Teeth were obtained from a pool of extracted teeth stored at
–4°C immediately after extraction. Informed consent was ob-
tained from the donors. According to the local ethics commit-
tee, an approval for irreversibly anonymized use of these by-
pass products was not needed. Single-rooted maxillary and
mandibular permanent premolars were chosen for the study.
Inclusion criteria were a single root with a mature apex and a
single round-to-oval root canal; exclusion criteria were an exist-
ing root canal filling, deep coronal caries, obvious translucency
of the root, a pronounced root curvature, and an immature apex.

The teeth were defrosted for 4 h at 4°C. They were then stored
in 3% (w/v) sodium hypochlorite for 1 h for removing the pe-
riodontal tissue. After access cavity preparation, the working
length was established using a size 10.02 file and was de-
defined to be 0.5 mm shorter than the length after first apical
visibility of the instrument. After establishing a glide path,
canals were negotiated with rotating instruments up to size
40 (ProTaper Universal F4, Maillefer, Ballaigues, Switzerland).
The irrigation solution used during instrumentation was 3%
Sodium hypochlorite, followed by a final rinse with 2 ml 17%
(w/v) EDTA. After closing the apex with glass ionomer cement
(Chemfil Rock, Dentsply, Tulsa, OK, USA), teeth were stored in
deionized water until further use.

Effect of DNase treatment on bacteria adhered to the
extracted teeth
Effect of DNase treatment in extracted teeth was assessed us-
ing a two-phase crossover design. For adhesion experiments,
28 teeth were randomly assigned to a treatment order and

EUROPEAN JOURNAL OF DENTAL IMPLANTOLOGY

18:82-6

EUR Endod J 2018; 2: 82-6

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autoclaved. Root surfaces were sealed with nail varnish, and canals were dried with sterile paper points (Protaper Universal F4, Maillefer, Ballaigues, Switzerland). E. faecalis suspensions were mixed with equal volumes of DNase I (final concentration 50 Kunitz/mL; 5 mM MgCl₂, in 0.9% NaCl) or heat-inactivated DNase I (100°C, 30 min) and injected into the root canals (n=56) using sterile syringes (gage 27, BD Microlance, Franklin Lakes, NJ, USA). Teeth were incubated for 1 h at 35°C under anaerobic conditions in a humid chamber. Then all teeth were thoroughly rinsed with 5 mL of sterile 0.9% NaCl using sterile syringes for removing non-adherent cells. Circumferential filing was performed (1 cm height of stroke, 2 strokes/s) with Hedström files (30.02, VDW, Munich, Germany) for 30 s. Thereafter, 10 μL of 0.9% NaCl was sampled from the canals using sterile paper points (Protaper Universal F4) that were then transferred to 1 mL of 0.9% sterile NaCl and vortexed for 2 min. The resulting suspension was serially diluted and plated on blood agar plates in sextuplicates. After 2 days of incubation at 35°C, colony forming units (CFU) were determined. Between two experimental phases, teeth were cleaned thoroughly, autoclaved again, and the nail varnish was applied again. Sterility after autoclaving was assured by incubating teeth other than the ones used for the experiment with sterile TSB and cultivating the broth on blood agar plates.

Effect of DNase treatment on biofilm dispersal in extracted teeth

For biofilm experiments, nine teeth were randomly assigned to a treatment order and autoclaved. Suspensions of E. faecalis were injected into the canals (n=18), as described above, and incubated at 35°C for 24 h. Thereafter, teeth were carefully dried with sterile paper points, and DNase I or heat-inactivated DNase I (50 Kunitz/mL; 5 mM MgCl₂, in 0.9% NaCl) was injected with sterile syringes and left to incubate for 1 h at 35°C under anaerobic conditions. Teeth rinsing, circumferential filing, sampling, serial dilution, and E. faecalis cultivation were performed as described in the adhesion experiments.

Statistical analysis

For the experiments in 96-well plates, average areas covered by bacteria were calculated for DNase- and heat-inactivated DNase-treated wells. Normal distribution was verified using QQ-plots and Shapiro–Wilk normality tests. One-tailed t-tests were performed for assessing whether the area was lower after DNase treatment. Calculations were performed using R (www.r-project.org). Based on results on bacterial adhesion in 96-well plates, a sample size of 28 was calculated for detecting a 25% treatment difference (α=0.05; 1-β=0.8) in teeth. Because no effect of DNase was observed on established biofilms in 96-well plates, a sample size of 28 was calculated for detecting a 25% treatment difference (α=0.05; 1-β=0.8) in teeth. Because no effect of DNase was observed on established biofilms in 96-well plates, a sample size of 28 was calculated for detecting a 25% treatment difference (α=0.05; 1-β=0.8).

RESULTS

After 1 h of adhesion in 96-well plates, most but not all E. faecalis cells showed overlapping signals from TOTO®-1 (green) and SYTO® 60 (red). In some areas, eDNA was observed as cloud-like structures without SYTO® 60 signals (white arrowheads; Fig. 1a). If both stains have equal access to DNA targets, TOTO®-1 outcompetes SYTO® 60 because of its higher binding affinity. Therefore, double staining of cells with both stains can be explained by the fact that the cell membrane is partially permeable to TOTO®-1 or it can be an indication of eDNA being present on the bacterial cell surface. As no green fluorescence was observed on DNase-treated cells (Fig. 1b), the signal is probably due to eDNA. Cloudlike eDNA structures were also present in 24-h-old biofilms of E. faecalis, but only few cells showed distinct cell surface staining (Fig. 1c). In DNase-treated 24-h-old biofilms, TOTO®-1 staining revealed considerably fewer cloud-like eDNA patches (Fig. 1d).

To test whether eDNA present on and in between cells of E. faecalis had an impact on bacterial attachment, the area of adhering cells in the presence of DNase or heat-inactivated DNase was quantified. The area covered by cells after 1 h of incubation with DNase was 54.6%±5.9% (SD) lower that that...
by cells under control treatment with heat inactivated DNase (P<0.05). Treatment with DNase, thus, hampered adhesion of \textit{E. faecalis} to 96-well plates (Fig. 2a and b). In extracted teeth, treatment with DNase resulted in fewer cells of \textit{E. faecalis} adhering to the dentin wall after 1 h of adhesion, but the difference with the control treatment with heat-inactivated DNase was not statistically significant (P=0.14; Fig. 3).

The area covered by bacteria in 24-h-old biofilms in 96-well plates did not decrease as a result of DNase treatment (−15.0%±9.8%; P=0.96). Representative images are shown in Fig. 2c and d. Likewise, DNase treatment of 24-h-old \textit{E. faecalis} biofilms did not reduce the number of CFU recovered from dentine samples (P=0.98; Fig. 3). As the recovered CFU were nearly identical for DNase and heat-inactivated DNase treatments, the experiments were stopped after nine teeth had undergone both treatments.

**DISCUSSION**

The importance of eDNA for bacterial biofilm formation, including biofilms of \textit{E. faecalis}, is uncontested; dispersing biofilms by enzymatic treatment of matrix components may be a promising therapeutic approach in different medical fields (9). Our data, however, do not support the sole use of DNase as an anti-biofilm agent during root canal treatment.

It has previously been shown that the number of \textit{E. faecalis} cells adhering to dentin blocks is reduced in the presence of DNase (15), albeit with less than 1 log reduction. In this study, we found that DNase treatment decreased adhesion of \textit{E. faecalis} to the polymer surface of microwell plates (P<0.05), but not to the surface of root canals (P=0.14; Fig. 2 and 3). Root canal anatomy is complex, and although treatment was performed in single-rooted premolars with one canal, it is conceivable that the enzyme did not exert its action in all sampled areas or that the rinsing procedure was less effective compared with treatment on dentine blocks. Furthermore, differences in the choice of strains (\textit{E. faecalis} DSM 20478 vs. ATCC 29212), the employed medium (TSB vs. brain heart infusion), the concentration of DNase (50 Kunitz/mL vs. 220 Kunitz/mL), and in the sampling procedure (30 s of circumferential filing vs. 5 min of ultrasonication) may explain the different results observed by Li et al. (15). In the present study, root canals were irrigated with sodium hypochlorite and EDTA during instrumentation, and no inactivation of sodium hypochlorite with sodium thiosulfate was performed prior to bacterial inoculation and DNase treatment. While both irrigation solutions can compromise enzyme activity of DNase, an inhibition was unlikely in the present setup because the teeth were stored in demineralized water for several weeks and autoclaved before DNase treatment. Finally, differences in root canal anatomy between individual teeth are unlikely to influence the treatment results as all teeth were treated both with active and heat-inactivated DNase. Changes in the canal morphology produced by 30 s of manual circumferential filing in between treatments may be considered negligible.

After 24 h of undisturbed biofilm formation, no effect of a 1 h DNase treatment could be observed in 96-well plates and extracted teeth (Fig. 2 and 3). Similarly, Li et al. reported lower effects of DNase treatment after 12, 24, and 48 h compared to 1 h, although DNase was present during the entire experiment (15). These results are in accordance with those of other studies that have demonstrated that the contribution of eDNA

![Figure 2](image-url)  
(a-d) Effect of DNase treatment on Enterococcus faecalis in 96-well plates. Cells of \textit{E. faecalis} were inoculated into 96-well plates and treated for 1 h with heat-inactivated DNase (HI DNase) (a and c) or DNase (b and d) during adhesion (a and b) or after 24 h of biofilm formation (c and d). Bacteria were stained with SYTO® 9 and quantified in confocal microscopy images. DNase treatment hampered adhesion of \textit{E. faecalis} after 1 h compared with that observed with the control treatment (P<0.05), but did not reduce the amount of biofilm grown after 24 h (P=0.98). Bars=100 μm

![Figure 3](image-url)  
Effect of DNase treatment on Enterococcus faecalis in teeth. Cells of \textit{E. faecalis} were inoculated into extracted teeth and treated for 1 h with heat-inactivated DNase (HI DNase) or DNase during adhesion or after 24 h of biofilm formation. The number of adhering cells was quantified using CFU counts. No statistically significant difference was found between DNase and control treatment. Error bars=95% confidence intervals
to biofilm stability is predominantly observed in the initial stages of biofilm formation (1, 4, 11, 17). Staining with TOTO-1 showed that DNase treatment of 24-h-old biofilms in 96-well plates removed most of the cloud-like eDNA patches in the biofilms (Fig. 1). It is, thus, unlikely that the treatment effect ceased after 24 h because of penetration problems or because eDNA was protected from enzymatic digestion. Most likely, matrix components other than eDNA secure biofilm stability at this stage of biofilm development (18).

The present study mimicked a clinical setting only to some degree. Single-rooted teeth with straight and thus easy-to-treat canals were chosen, and monospecies biofilms were grown for no more than 24 h. In a clinical setting, and particularly in retreatment cases, biofilms are mature and typically comprise multiple species (19); often, the intricate root anatomy renders mechanical and chemical debridement more difficult. Even under the chosen simplified conditions, though, DNase treatment had little impact on adhesion and biofilm stability of E. faecalis. Prior to sampling, the root canals were flushed with copious amounts of NaCl to remove planktonic organisms and predominantly sample bacteria that adhered firmly to the canal walls (20, 21). Sampling was, however, limited to areas that were accessible to mechanical debridement with 30.02 Hedström files and may not have reached all parts of the root canal (22). Still, there is little reason to believe that the effect of DNase treatment is more pronounced in sites inaccessible to mechanical cleaning. It is conceivable that the matrix-degrading effect of DNase enhances the penetration of antiseptic enzymes influences biofilm development. J Bacteriol 2008; 190(16):5690–8.

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CONCLUSION

In conclusion, this study shows that DNase treatment hampers adhesion of E. faecalis under in vitro conditions. However, DNase has no effect, as a therapeutic agent to disturb E. faecalis colonization in an ex vivo model.

Disclosures

Conceived and designed the experiments: S.S., J.G., K.W.N., R.L.M.
Performed the experiments: S.S., K.W.N., J.G.
Analyzed the data: S.S., M.V.
Wrote and approved the paper: S.S., J.G., K.W.N., M.V., R.L.M.
Conflict of Interest: The authors deny any conflict of interest.
Ethics Committee Approval: Not required.
Peer-review: Externally peer-reviewed.
Financial Disclosure: This work was supported by the Carlsberg foundation (grant number 2011-01-0567) and Danish Council for Independent Research (0602-021308). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors deny any conflicts of interest related to this study.

Authorship contributions: Concept – S. S., K. W. N.; Design – S. S., K. W. N., J. G.; Supervision – S. S., R. L. M.; Fundings – S. S., K. W. N., R. L. M.; Materials – S. S.; Data collection &/or processing – S. S., J. G., M. V.; Analysis and/or interpretation – S. S., K. W. N., M. V.; Literature search – S. S., J. G.; Writing – S. S., K. W. N.; Critical review – S. S., K. W. N., R. L. M., J. G., M. V.