

# Reduction of dual-species biofilm after sonic- or ultrasonic-activated irrigation protocols: A laboratory study

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## Abstract

**Aim:** To evaluate the antibacterial effect of sonic- and ultrasonic-activated irrigation on bacterial reduction of a dual-species biofilm in root canals compared to nonactivated irrigation in a laboratory study.

**Methodology:** Two hundred and forty extracted human single-rooted maxillary anterior teeth were divided into two main groups (G,  $n = 120$ ) according to the initial preparation size of the root canal (G1: size 25, 0.06 taper, G2: size 40, 0.06 taper). Root canals were inoculated with *Enterococcus faecalis* and *Streptococcus oralis*. After 5 days, G1 received combined instrumentation (up to size 40, 0.06 taper) and irrigation/activation, whereas G2 received solely irrigation/activation protocols. In both groups, irrigation was performed with sodium hypochlorite (NaOCl 1%) or physiological saline (NaCl 0.9%), using nonactivated syringe irrigation, sonic activation (2 x 30 s) or ultrasonic activation (2 x 30 s). Logarithmic reduction factors (LRFs) of colony-forming units were analysed separately for dentine-adherent and planktonic bacteria immediately after irrigation/activation protocols (time-point 1) or after 5 days of further incubation (time-point 2) by analysis of variance (ANOVA) and *post hoc* tests (Tukey's HSD, t-test). The significance level was set at 0.05.

**Results:** In G1 subgroups (combined instrumentation with irrigation/activation), LRFs were significantly affected by the applied irrigation solution ( $p < .0001$ ), but not by the activation method ( $p > .05$ ; ANOVA). In G2 subgroups (solely irrigation/activation), both, irrigant solution and activation, significantly affected LRFs ( $p < .0001$ , ANOVA). Sonic activation resulted in significantly higher LRFs than ultrasonic activation ( $p < .0001$ ) which had significantly greater reductions than nonactivated irrigation ( $p < .05$ ; Tukey's HSD). At T2, strong bacterial regrowth was observed in all groups; however, a significant bacterial reduction was detected for factors instrumentation, irrigant solution and activation ( $p < .0001$ ; ANOVA). Similar LRFs were found for dentine-adherent and planktonic bacterial cells in all groups ( $r = 0.91$  at T1,  $r = 0.8$  at T2).

**Conclusions:** In this laboratory study on extracted maxillary anterior teeth high-frequency sonic activation resulted in a greater bacterial reduction compared to ultrasonic activation in groups receiving solely irrigation/activation protocols; however,

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irrigation using NaOCl and ultrasonic activation also contributed significantly to bacterial reduction compared to the control groups.

#### KEYWORDS

antibacterial effectiveness, nonactivated irrigation, root canal disinfection, sonic activation, ultrasonic activation

## INTRODUCTION

The main goal of root canal treatment is the prevention or treatment of apical periodontitis (Nair, 2004). To achieve this aim, the removal of vital or necrotic pulp tissue, microorganisms and their toxins from the root canal space is essential (Gu et al., 2009). Since more than 50% of the root canal walls remain untouched after instrumentation (Paqué et al., 2009), irrigation plays an important role to reduce the bacterial load to a subcritical level (Siqueira & Rôças, 2008). Manual conventional needle irrigation delivers endodontic irrigants no more than 0–1.1 mm beyond the needle tip, depending on flow rate, apical preparation size and taper as well as the design of the needle tip (Boutsioukis et al., 2010a, 2010b; 2010c). Consequently, complete removal of debris and smear layer from the root canal system is unlikely using a manual technique (Versiani et al., 2015).

To overcome these problems, activation of the irrigant has been suggested, with the aim to facilitate dispersion and replenishment of the solutions. The current gold standard is represented by passive ultrasonic irrigation (PUI), which is effective in both root canal disinfection and removal of the smear layer. This efficacy has been mainly attributed to cavitation (growth and subsequent implosion of liquid bubbles) and to an acoustic streaming effect created by the ultrasonic device (van der Sluis et al., 2007). However, a randomized clinical trial failed to confirm its efficacy in improving periapical healing of root canal-treated teeth compared to conventional needle irrigation (Liang et al., 2013). In addition, PUI has several drawbacks; when the oscillating tip touches the root canal wall, especially in curved root canals, the energy is reduced and the file movement constrained (Boutsioukis et al., 2013). Moreover, ultrasonic files are made of metal alloy that may lead to uncontrolled dentine removal when contacting the root canal wall (Lea et al., 2009; Retsas et al., 2016).

Sonic activation devices operate at lower frequencies of 1–10 kHz compared to ultrasonic activation, which operates at frequencies of 25–30 kHz. Sonic activation is associated with hydrodynamic phenomena through oscillation of smooth and highly flexible polymer tips (Gu et al., 2009). The sonic device, EDDY® (VDW), operates at a frequency of 6 kHz and the manufacturer claims that the oscillating movement triggers cavitation and acoustic

streaming enhancing root canal cleaning and antibacterial effectiveness.

Most of the published studies that evaluated the antibacterial efficacy of the high-frequency sonic irrigation device EDDY® used monospecies biofilm models comprising *Enterococcus faecalis* and revealed no significant differences compared to ultrasonic activation, but enhanced efficacy compared to manual conventional syringe irrigation (Al-Obaida et al., 2019; Eneide et al., 2019; Hage et al., 2019; Neuhaus et al., 2016). However, laboratory studies evaluating the reduction of dentine-adherent dual- or multi-species biofilms in root canals are scarce.

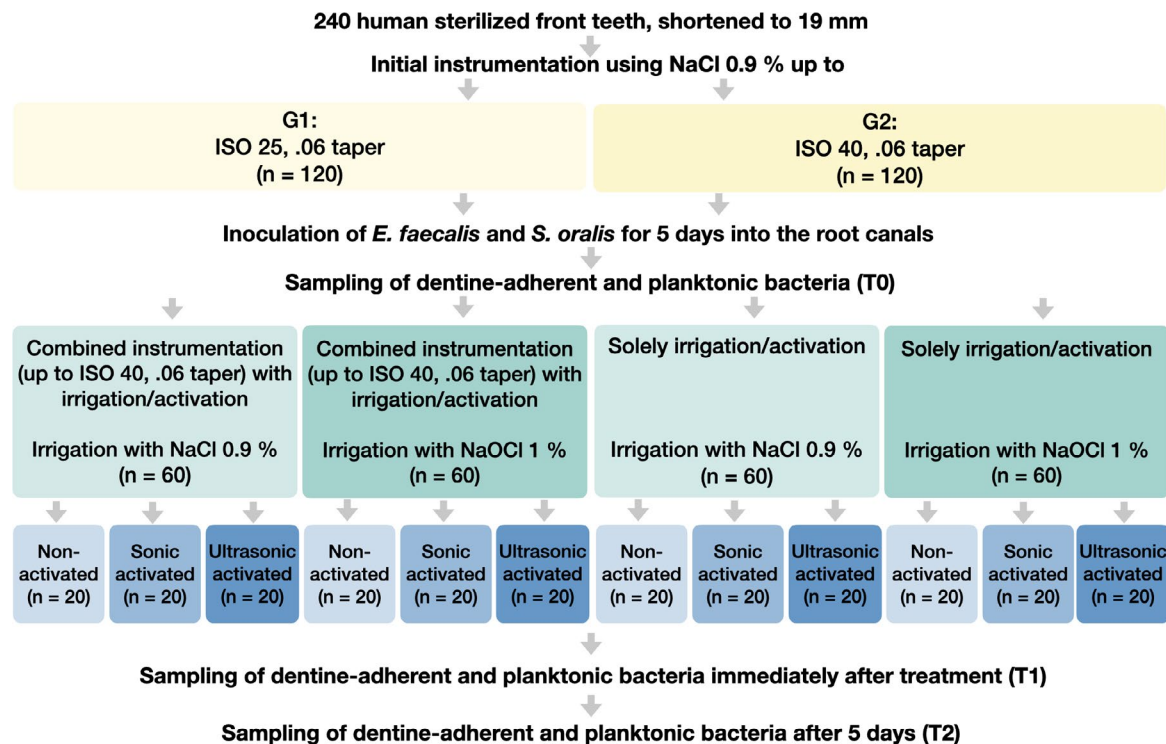
Consequently, the aim of the present study was to evaluate the reduction of intracanal dentine-adherent and planktonic bacteria of a dual-species laboratory biofilm in human root canals after sonic and ultrasonic irrigation. A nonactivated manual irrigation procedure was used as a reference for comparison. The null hypothesis of the present study was that bacterial reduction is not affected by the applied activation protocols.

## MATERIALS AND METHODS

Sample preparation, bacterial inoculation, root canal instrumentation, irrigation and activation as well as sampling and determination of bacteria were performed by one operator (N.K.). The study design is illustrated by a flowchart in Figure 1.

### Sample preparation

A total of 240 extracted human maxillary anterior teeth with straight root canals, without root canal fillings, root caries or restorations were obtained with written informed consent under an ethics-approved protocol (EA4/102/14) by the Ethical Review Committee of the Charité – Universitätsmedizin Berlin, Germany, and stored in 0.5% chloramine T solution prior to experimental use. The teeth were cleaned with ultrasonic scalers (SONICflex; KaVo), and subsequently plasma-sterilized (STERRAD® 100NX System; Cilag GmbH International). Crowns were removed and all roots were shortened to a uniform length of 19 mm using a diamond-cutting device (EXAKT



**FIGURE 1** Study design visualized by flow chart. Sample preparation, treatment procedure and sampling of dentine-adherent and planktonic bacteria are illustrated. G, group; n, number; NaCl, sodium chloride; NaOCl, sodium hypochlorite; T, time.

Trennschleifsystem 300 CL; EXAKT). The apical foramen was sealed using a self-etch adhesive system (FuturaBond DC; VOCO GmbH) and a resin composite (Spectrum<sup>TM</sup> 800; Denstply Caulk) according to the manufacturer's instructions. After covering the root surfaces with nail varnish (Maybelline; Color Show 60 Seconds), each root was embedded into closable cryo-tubes (Carl Roth) using epoxy resin (Technovit 4071; Heraeus Kulzer). At the canal orifice of each root, a cavity was prepared with a round diamond bur (Diamond sphere size 029 Komet; Gebr. Brasseler) to serve as a reservoir for the bacterial suspension. To achieve orientation points for subsequent dentine-adherent biofilm sampling, narrow marks were made at three locations of the coronal root surface for sampling at baseline (T0), after treatment (T1) and after 5 more days of incubation (T2), see Figure S1. The coronal third of all root canals was enlarged using Gates Glidden Burs sizes 4 and 5 (VDW).

Subsequently, samples were randomly divided into two main groups with 120 samples per group according to the preparation size prior to inoculation.

Root canals of group 1 (G1) were instrumented using a rotary NiTi shaping system up to size X2 (size 25, 0.06 taper) and root canals of group 2 (G2) up to size X4 (size 40, 0.06 taper; ProTaper Next; Dentsply Sirona). Thus, canals of G1 represent canals with a primary infection that require a combination of instrumentation and irrigation/

activation, whereas in canals of G2, the effects of solely irrigation/activation were evaluated without being masked by instrumentation.

Irrigation was performed using 5 mL of saline solution (NaCl 0.9%; Pharmacy Charité) after each change of file. Working length was established at 18 mm in all samples. Following the root canal preparation, the smear layer was removed using 5 mL of ethylenediaminetetraacetic acid (EDTA) 17% (CanalPro EDTA 17%; Coltene/Whaledent) for 2 min. EDTA was removed by irrigation with 10 mL of saline (NaCl 0.9%; Pharmacy Charité) as a final rinse. Subsequently, samples were plasma-sterilized again and stored in sterile boxes (50-mL Falcon tubes; Sarstedt) filled with sterile brain–heart infusion (BHI; SIFRIN), for a period of at least five days at 37°C. Sterility was indicated by clear media.

## Bacterial inoculation

Following sterilization, the root canals were inoculated with bacterial suspensions of *E. faecalis* (ATCC 29212) and *Streptococcus oralis* (ATCC 35037). A mixed inoculum of both bacterial species in the same proportions was injected into the root canal using a sterile needle and syringe (5-mL Syringe; BD Plastipak<sup>TM</sup>) until the root canals and the reservoir were filled completely. Samples

were incubated under anaerobic conditions for 5 days at 37°C, whilst fresh BHI and new atmosphere generating bags (AnaeroGen; Thermo Fisher Scientific Oxoid) were added every day.

## Instrumentation, irrigation and activation of the infected root canals

Root canals were treated with the following instrumentation and disinfection protocols inside a laminar flow hood (Mikrobiologische Sicherheitskabine; Bleymehl Reinraumtechnik): root canals of G1 were instrumented from size 25, 0.06 taper up to size 40, 0.06 taper (X4), with 1.5 mL of irrigant solution between files, and 4.5 mL after the last file. In G2, instrumentation up to size 40, 0.06 taper was already performed prior to bacterial inoculation; therefore, no more instrumentation was performed in the infected root canals. In each group, half of the samples were irrigated using 0.9% NaCl (Pharmacy Charité), whilst the other half was irrigated using 1% sodium hypochlorite (NaOCl (Pharmacy Charité). In the nonactivated groups (manual irrigation), the irrigants (10-mL volume each) were applied 1 mm before working length with a flow rate of 1 mL/10 s using a 30-gauge open-ended needle (NaVi Tip 30 ga; Ultradent Products) with medium pressure, along with a slight in-and-out movement. In the test groups, samples were either activated using ultrasonic activation with an IRRIS file (size 25; VDW) at 30% power, or sonic activation using a polyamide tip (size 25, 0.04 taper; EDDY<sup>®</sup>, VDW) coupled to an air scaler (SONICflex, intensity mode III; KaVo) with the tip applied 1 mm from working length. Two activation cycles per subgroup were conducted: first 4 mL of the irrigant was applied using a 30-gauge syringe needle, followed by an activation cycle for 30 s, a 3 mL rinse, a second activation cycle and a final rinse of 3 mL. In groups using NaOCl subsequent rinsing using 5 mL NaCl 0.9% was conducted to remove remnants of NaOCl.

## Sampling of dentine-adherent and planktonic bacteria and determination of colony-forming units

Sampling of bacteria was performed at three different time-points from each root canal: before treatment (T0), immediately after therapy (T1) and after 5 days of further incubation (T2). Prior to sampling, each root canal was dried using a sterile paper point and subsequently filled with sterile saline (0.9% NaCl). The sampling of planktonic bacteria from each canal was performed by inserting

one sterile paper point (size 25, 0.02 taper; VDW) until it was soaked with liquid to a mark of 20 mm, obtaining 5 µL of liquid sample. Each paper point was placed in 1995 µL BHI and vortexed for 30 s. After drying the root canal with paper points (size 40, 0.02 taper; VDW), sampling of dentine-adherent bacteria was performed by moving a Hedström file (size 25, 0.02 taper; VDW) from apical to coronal at the root canal wall with three vigorous pressured strokes. For each time-point, dentine was removed in one of the three previously marked regions of the canal wall (see Figure S1). Handles of the Hedström files were detached and the working ends were placed into cryo-tubes containing 50 µL of BHI and vortexed for 30 s.

Both planktonic and dentine-adherent bacterial samples were diluted serially before plating on culture plates (Columbia agar plates with 5% sheep blood; Heipha). All plates were incubated under anaerobic conditions for 3 days at 37°C before the colony-forming units (CFUs) per mL were determined.

## Validation of the biofilm by Fluorescence in situ hybridization (FISH)

After biofilm growth, three teeth were fixed in 4% paraformaldehyde, embedded in cold polymerizing resin (Technovit 8100; Heraeus Kulzer) and sectioned with a saw microtome (Ernst Leitz GmbH). Slices of 1 mm were decalcified in 17% EDTA acid for 21 days, followed by digital X-ray analysis to confirm complete decalcification. Slices were then re-embedded in resin (Technovit 8100), thin sections of <2 µm were cut on an ultramicrotome (Ultracut E; Reichert Jung Optische Werke AG) and mounted on coated glass slides (Polysine; Menzel-Gläser). Details of the procedure have been described previously (Dige et al., 2014; Hoedke et al., 2018).

Probes STR405 (5'-TAG CCG TCC CTT TCT GGT-3'), ENF191 (5'-GAA AGC GCC TTT CAC TCT TAT GC-3') and EUB338 (5'-GCT GCC TCC CGT AGG AGT-3') were employed to target *S. oralis*, *E. faecalis* and total bacteria respectively. STR405 was 5'-end-labelled with Alexa488, ENF191 with Cy5 and EUB338 with Cy3 (IBA). FISH was performed according to the protocol described in Dige et al. (2009). Fixed cells of *S. oralis* and *E. faecalis* were included in the experiments and served either as positive or negative controls for the specific probes respectively (see Figure S2).

Following FISH, tooth sections were imaged with a confocal microscope (Zeiss LSM 700) equipped with a 63x objective (alpha Plan-Apochromat, Zeiss). To avoid cross talk between channels, Alexa488/Cy5 and dentine autofluorescence/Cy3 were excited sequentially.

## Sample size calculation

This study included three treatment modalities, i.e., instrumentation, irrigant solution and activation method, which resulted in 12 treatment groups. Sample size calculation was conducted for multi-way ANOVA with more than one category of interest. As widely accepted for experimental studies, the probability for  $\alpha$ -error was set at 0.05 and the power at 0.8. Based on the results of a previous study (Hoedke et al., 2018), an effect size of 0.31 was calculated leading to a total sample size of 185 (G\*Power; Heinrich-Heine-Universität Düsseldorf, Germany; Faul et al., 2007). To achieve balanced groups, sample size was adjusted in the present study to 240 resulting in  $n = 20$  per group.

## Statistical analysis

Colony-forming units counts of dentine-adherent and planktonic bacteria for both species were log transformed and logarithmic reduction factors (LRF) were calculated between T0 and T1 (LRF1) and T0 and T2 (LRF2). Statistical analysis was stratified by sampling time (T1 immediately after therapy, T2 after five additional days of incubation) and the location of the bacteria (dentine-adherent bacteria, planktonic bacteria). Three-way ANOVA was carried out to determine the effect of instrumentation (factor 1), irrigation solution (factor 2) and of additional activation (factor 3) on LRF. Due to multiple interactions in our results, we carried out a second two-way ANOVA, stratified for instrumentation to identify main effects, with irrigation solution and additional activation as factors. *Post hoc* tests (Tukey's HSD, t-tests) were applied to assess differences between groups. The significance level ( $\alpha$ ) was set at 0.05. Pearson correlation was performed to compare

the amount of dentine-adherent and planktonic bacteria in each sample. All analyses were performed using SPSS statistics 25 (IBM).

## RESULTS

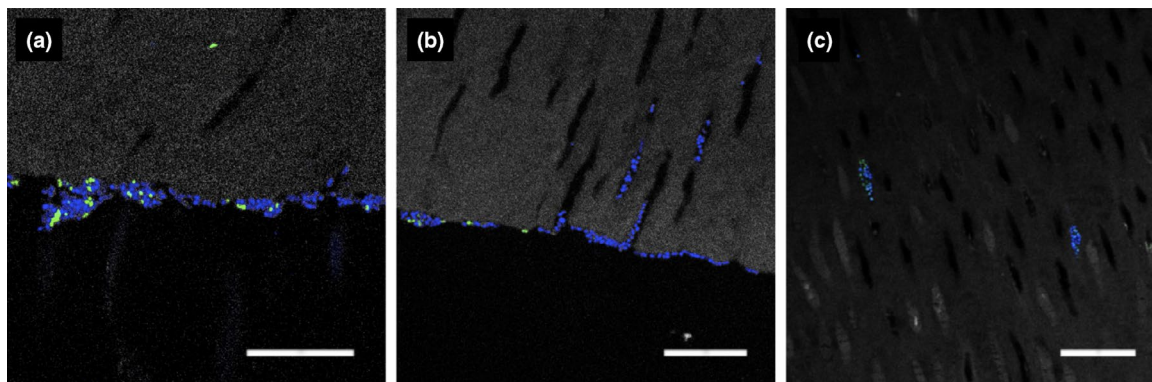
### Fluorescence *in situ* hybridization

The probes proved to be species specific under the chosen experimental conditions (see Figure S2). Biofilm formation was validated by FISH, demonstrating the presence of thin biofilms dominated by *E. faecalis* in all investigated specimens. The cells were attached to the root canal walls, and both species invaded dentinal tubules in some locations (see Figure 2a-c).

### Reduction of dentine-adherent and planktonic bacteria

Descriptive data on bacterial loads for baseline (T0), immediately after therapy (T1) and after 5 days of further incubation for dentine-adherent and planktonic bacteria are provided in Tables S1 and S2.

Immediately after treatment (T1), bacterial reduction for dentine-adherent and planktonic bacteria was significantly affected by instrumentation, irrigant solution and the applied activation method ( $p < .0001$ ). Significant interactions between instrumentation and the activation method ( $p < .03$ ) and between the irrigant solution and the activation method ( $p < .03$ ) could be observed for both, planktonic and dentine-adherent bacteria (three-way ANOVA). Due to multiple interactions, the analyses were stratified for G1 (combined instrumentation and irrigation/activation) and G2 (solely irrigation/activation).



**FIGURE 2** Fluorescence *in situ* hybridization (FISH) of biofilms in root canal sections. FISH with specific probes for *Streptococcus oralis* (displayed in green) and *Enterococcus faecalis* (displayed in blue) had thin biofilms in all examined specimens. Cells were firmly attached to the root canal dentine (a, b) and invaded dentinal tubules in some locations (b, c). Dentine autofluorescence is displayed in grey tones. Bars = 20  $\mu\text{m}$ .

In G1, bacterial reduction was significantly affected by the applied irrigation solution ( $p < .0001$ ), but not by the activation method ( $p > .05$ , two-way ANOVA, Table 1).

In G2, bacterial reduction was affected by the applied irrigation solution as well as by the activation method and an interaction between both factors was observed ( $p < .0001$ , two-way ANOVA). Sonic-activated irrigation resulted in significantly greater bacterial reduction compared to ultrasonic-activated irrigation ( $p < .0001$ ), which, in turn, resulted in significantly greater bacterial reduction when compared to nonactivated irrigation ( $p < .002$ , Tukey's HSD, Table 2).

Strong bacterial regrowth was observed in all groups at T2, resulting in mean LRFs of dentine-adherent bacteria ranging from  $-0.5$  to  $1.2 \log_{10}$  steps and for planktonic bacteria ranging from  $-0.4$  to  $1.2 \log_{10}$  steps. Statistically significant effects on bacterial reduction were detected for factors instrumentation and irrigant solution ( $p < .0001$ ). LRFs from dentine-adherent bacteria were significantly affected by activation ( $p = .001$ ), whilst no significant effect for planktonic cells was observed ( $p = .3$ ; Tables 1 and 2).

With regard to the different sampling methods, there were strong correlations between dentine-adherent and planktonic LRFs ( $r_{(LRF1)} = 0.91$ ,  $r_{(LRF2)} = 0.8$ ).

## DISCUSSION

The null hypothesis of the present study was partially rejected; immediately after treatment (T1), groups that received solely irrigation/activation (G2) demonstrated significantly greater bacterial reductions for sonic activation compared to ultrasonic activation, and both activation methods were more effective compared to nonactivated groups (manual irrigation) when using 1% sodium hypochlorite. These results are in contrast to previous studies that revealed a comparable antimicrobial effect of sonic activation using the EDDY<sup>®</sup> device and ultrasonic activation against *E. faecalis* monospecies biofilms (Al-Obaida et al., 2019; Eneide et al., 2019; Hage et al., 2019). Only one previous study reported an increased antimicrobial efficacy of the sonic device EDDY<sup>®</sup> when using sodium chloride as an irrigant in straight as well as in curved canals (Neuhaus et al., 2016). Conflicting results may be attributed to variations in final instrumentation sizes prior to bacterial inoculation and sampling methods, as well as different bacteria in the biofilm models.

Although the driving frequency of the sonic activation device EDDY<sup>®</sup> (6 kHz) is markedly lower than that of an ultrasonic file (25–30 kHz), sonic activation was more effective compared to ultrasonic activation immediately after treatment in groups that received solely irrigation/

activation in the present study. However, after further incubation or in groups with combined instrumentation and irrigation/activation, this difference was not observed. Ultrasonic activation has been reported to result in microacoustic streaming and cavitation of the surrounding irrigation solution (van der Sluis et al., 2007), whereas these phenomena could not be detected around sonically oscillating instruments, because the movement of the tip appeared to be too slow and below the cavitation threshold (Macedo et al., 2014; Swimberghe, De Clercq et al., 2019b). On the other hand, the displacement amplitude of the EDDY<sup>®</sup> tip (350  $\mu\text{m}$ ) has been described as being greater compared to an ultrasonic-activated tip (75  $\mu\text{m}$ ), and a three-dimensional tip movement of the EDDY<sup>®</sup> has been suggested (Swimberghe, De Clercq et al., 2019b). Furthermore, application of the EDDY<sup>®</sup> tip is accompanied by an up-and-down movement, whereas the IRRI S file is kept steady. Both the increased amplitude and the inherent up-and-down movements may contribute to an enhanced fluid movement inside the root canal. The EDDY<sup>®</sup> tip has a size of 20 with a 0.05 taper, whereas the tip size of the IRRI S file was 25 with a parallel shape. These differences in size and taper could also possibly contribute to the differences in performance. However, the EDDY<sup>®</sup> tips are only available in one size; consequently comparison with other tapers or sizes is currently not possible. This being said, it must be emphasized that none of the applied methods was able to completely remove the biofilm from the root canals in combination with NaOCl 1%, as shown by the strong bacterial regrowth in all groups after five days of further incubation.

A previous randomized clinical trial failed to show superiority of ultrasonic activation compared to manual syringe irrigation when treating mandibular premolars (Liang et al., 2013). This was confirmed by a recent systematic review that included two additional clinical studies analysing bacterial reduction after activation protocols. The results were inconclusive, one study reported higher bacterial reduction for irrigant activation, whereas the other study did not report significant differences (Silva et al., 2019). Since clinical trials are time consuming and expensive, and endodontic outcome studies are challenging, the use of laboratory biofilm models to evaluate the antibacterial effectiveness of various disinfection protocols and devices is common practice. These models should, on the one hand, reflect the clinical situation closely, and on the other hand, provide sufficient feasibility, reproducibility and standardization (Swimberghe, Coenye et al., 2019). To facilitate standardization of the samples, straight root canals of incisors without curvatures or irregular canal structures were used. The uncomplicated canal morphology may explain why activation did not improve antibacterial effectiveness when applied

**TABLE 1** Logarithmic reduction factors for dentine-adherent and planktonic bacteria for groups with combined instrumentation and irrigation/activation (G1)

| G1   | Combined instrumentation and irrigation/activation |                                   |                         |                            |                        |                                 |          |
|--|--|-----------------------------------|-------------------------|----------------------------|------------------------|---------------------------------|----------|
|  | Activation method                                  | Nonactivated irrigation (control) |                         | Sonic-activated irrigation |                        | Ultrasonic-activated irrigation |          |
|  |  | NaCl 0.9%                         | NaOCl 1%                | NaCl 0.9%                  | NaOCl 1%               | NaCl 0.9%                       | NaOCl 1% |
| <b>Irrigation Solution</b>                 |  |                                   |                         |                            |                        |                                 |          |
| Dentine-adherent LRF 1 (T0–T1) (Mean ± SD) | 3.3 ± 1.7 <sup>a</sup>                             | 6.2 ± 0.8 <sup>b</sup>            | 3.8 ± 1.9 <sup>a</sup>  | 6.5 ± 0.1 <sup>b</sup>     | 3.8 ± 2.4 <sup>a</sup> | 6.4 ± 0.1 <sup>b</sup>          |          |
| Dentine-adherent LRF 2 (T0–T2) (Mean ± SD) | 0.3 ± 0.5 <sup>a</sup>                             | 0.8 ± 0.2 <sup>b</sup>            | −0.1 ± 0.4 <sup>a</sup> | 1.2 ± 0.1 <sup>b</sup>     | 0.5 ± 0.3 <sup>a</sup> | 0.9 ± 0.6 <sup>b</sup>          |          |
| Planktonic LRF 1 (T0–T1) (Mean ± SD)       | 3.1 ± 1.5 <sup>a</sup>                             | 6.0 ± 1.1 <sup>b</sup>            | 3.7 ± 1.8 <sup>a</sup>  | 6.5 ± 0.1 <sup>b</sup>     | 3.5 ± 1.8 <sup>a</sup> | 6.5 ± 0.3 <sup>b</sup>          |          |
| Planktonic LRF 2 (T0–T2) (Mean ± SD)       | 0.0 ± 0.3 <sup>a</sup>                             | 0.9 ± 0.3 <sup>b</sup>            | −0.1 ± 0.4 <sup>a</sup> | 1.2 ± 0.1 <sup>b</sup>     | 0.1 ± 0.3 <sup>a</sup> | 0.9 ± 0.8 <sup>b</sup>          |          |

Abbreviations: CFUs, colony-forming units; G, group; LRF, logarithmic reduction factor; NaCl, sodium chloride; NaOCl, sodium hypochlorite; SD, standard deviation; T0, baseline; T1, immediately after therapy; T2, 5 days after therapy.

Superscript letters indicate significant differences between irrigation groups (two-way ANOVA, t-test).

**TABLE 2** Logarithmic reduction factors for dentine-adherent and planktonic bacteria for groups with solely irrigation and activation (G2)

| G2   | Solely irrigation/activation |                                   |                         |                            |                         |                                 |          |
|--|------------------------------|-----------------------------------|-------------------------|----------------------------|-------------------------|---------------------------------|----------|
|  | Activation method            | Nonactivated irrigation (control) |                         | Sonic-activated irrigation |                         | Ultrasonic-activated irrigation |          |
|  |                              | NaCl 0.9%                         | NaOCl 1%                | NaCl 0.9%                  | NaOCl 1%                | NaCl 0.9%                       | NaOCl 1% |
| <b>Irrigation Solution</b>                 |                              |                                   |                         |                            |                         |                                 |          |
| Dentine-adherent LRF 1 (T0–T1) (Mean ± SD) | 1.9 ± 0.5 <sup>a</sup>       | 3.9 ± 1.5 <sup>b,1</sup>          | 2.3 ± 0.2 <sup>a</sup>  | 6.5 ± 0.1 <sup>b,2</sup>   | 2.3 ± 0.3 <sup>a</sup>  | 4.9 ± 1.1 <sup>b,3</sup>        |          |
| Dentine-adherent LRF 2 (T0–T2) (Mean ± SD) | 0.1 ± 0.5 <sup>a</sup>       | 0.9 ± 0.4 <sup>b</sup>            | −0.5 ± 0.1 <sup>a</sup> | 0.9 ± 0.2 <sup>b</sup>     | 0.0 ± 0.7 <sup>a</sup>  | 1.0 ± 0.3 <sup>b</sup>          |          |
| Planktonic LRF 1 (T0–T1) (Mean ± SD)       | 2.1 ± 0.3 <sup>a</sup>       | 3.8 ± 1.3 <sup>b,1</sup>          | 2.2 ± 0.4 <sup>a</sup>  | 6.6 ± 0.2 <sup>b,2</sup>   | 2.3 ± 0.2 <sup>a</sup>  | 5.0 ± 1.1 <sup>b,3</sup>        |          |
| Planktonic LRF 2 (T0–T2) (Mean ± SD)       | −0.2 ± 0.2 <sup>a</sup>      | 0.7 ± 0.3 <sup>b</sup>            | −0.4 ± 0.2 <sup>a</sup> | 1.0 ± 0.2 <sup>b</sup>     | −0.2 ± 0.3 <sup>a</sup> | 0.8 ± 0.4 <sup>b</sup>          |          |

Abbreviations: CFUs, colony-forming units; G, group; LRF, logarithmic reduction factor; NaCl, sodium chloride; NaOCl, sodium hypochlorite; SD, standard deviation; T0, baseline; T1, immediately after therapy; T2, 5 days after therapy.

Superscript letters indicate significant differences between irrigation groups (two-way ANOVA, t-test).

Superscript numbers indicate significant differences between activation groups.

in combination with instrumentation; however, a superior effect occurred when solely irrigation and activation were applied in infected canals sized 40, 0.06 taper. The greater efficiency of ultrasonic, and especially sonic activation (LRF > 6) may also contribute to successful disinfection in more challenging clinical scenarios.

*Enterococcus faecalis* is frequently isolated from root canals with persisting apical pathosis, but it is not one of the most dominant species during primary infection (Rôças et al., 2008). Its resistance against inhospitable conditions and its fast growth render the organism easy to identify and cultivate. Moreover, its ability to resist long-term starvation (Hartke et al., 2002) and to penetrate deeply into dentinal tubules facilitates its use in endodontic biofilm experimental models. Oral streptococci are

primary dentine colonizers *in vivo* and bind to type-I collagen with the help of cell surface adhesins (Love et al., 1997). Therefore, they were selected as the second strain of the dual-species biofilm model for *ex vivo* inoculation of human root canals. Both *E. faecalis* and *S. oralis* are avid biofilm formers, frequently isolated from endodontic infections (Zandi et al., 2018) and have been associated with specific virulence traits (Lew et al., 2015). Both organisms were previously employed successfully in a three-species model with *Prevotella intermedia* (Hoedke et al., 2018), and only *E. faecalis* and *S. oralis* were shown to invade dentinal tubules. In addition, dual-species biofilms have been reported to be more resistant against NaOCl treatment compared to monospecies biofilms (Ozok et al., 2007), but they may not match the complexity and virulence of *in*

*vivo*-grown biofilms (Ordinola-Zapata et al., 2014). A dual-species model was selected as an appropriate compromise that provided some bacterial interaction but still allowed for sufficient standardization and rapid identification of the involved species.

Fluorescence *in situ* hybridization experiments confirmed that both strains inoculated into the teeth adhered firmly and formed biofilms inside the root canals. Most importantly, both organisms invaded dentinal tubules, which support the validity of the experimental setup as a model for endodontic infections. The use of FISH is, however, not suitable for quantification of bacterial cells in intracanal biofilms, as parts of the biofilm may be removed during sample processing (decalcification, sectioning).

Microbiological culturing methods were applied after bacterial sampling using either paper points (planktonic bacteria) or Hedström files (dentine-adherent bacteria), allowing determination of the number of CFUs in each sample as outcome measure in the present study. CFU counts are a frequently applied and universally accepted method for comparison between various disinfection modalities (Swimberghe, Coenye et al., 2019). Interestingly, both sampling methods (paper point and Hedström files) showed high correlations for the number of recovered CFUs. Hence, the amount of planktonic cells isolated from root canals may be indicative of the amount of dentine-adherent bacteria and vice versa. It may be assumed that the sampling methods described cover the detection of planktonic and superficial dentine-adherent bacteria which can be found within the root canal and on the root canal walls. Bacteria that are located in deeper parts of the dentinal tubules may not have been detected by the sampling strategy, since it is not possible to precisely define the depth of tubules, where bacteria were sampled using Hedström files. Nevertheless, all bacterial sampling procedures as well as endodontic procedures were performed by the same operator to standardize experimental conditions. However, undetected deep penetration of cells by the applied method could be the reason why no CFUs were identified in some of the samples at T1, whereas high CFU counts were observed at T2 in all samples. Alternatively, subpopulations of the cells in the biofilms may have been viable, but nonculturable immediately after treatment due to their low metabolic activity. The pronounced bacterial regrowth in all samples after 5 days of further incubation illustrates clearly, how difficult it is to achieve complete eradication of all microorganisms inside root canals, despite the use of a simplified laboratory-based biofilm model.

The present study used 1% NaOCl, which is a low concentration compared to other laboratory studies on antibacterial effectiveness (Ordinola-Zapata et al., 2014; Zeng et al., 2018). High NaOCl concentrations *in vitro*

may decrease the sensitivity of experiments in discerning differences of activation protocols (Zeng et al., 2018), and a recent randomized controlled trial did not demonstrate significant differences in healing rates when using either 1% or 5% NaOCl (Verma et al., 2019), proving the antibacterial effectiveness of low NaOCl concentrations.

No inactivation of NaOCl using sodium thiosulphate was performed in the present study, consequently a so-called carry-over effect of NaOCl inside the canal or the agar plate cannot be excluded (Hecker et al., 2013). However, data on the carry-over effect of NaOCl appear to be controversial; in a bovine root canal model infected with *E. faecalis*, no noticeable carry-over effect of NaOCl 4% was detected, possibly due to a dilution of the solutions after irrigation (Rossi-Fedele et al., 2010). Other authors speculate that the carry-over effect is negligible up to a NaOCl concentration of 3% (Muhammad et al., 2014; Rossi-Fedele et al., 2010). Even if the 'carry-over effect' was partly responsible for the decreased bacterial counts in NaOCl groups, the results for G2 demonstrate a clear difference between nonactivated and activated groups, which is valid since all groups were irrigated with the same volume of hypochlorite and were sampled with the same methods and effects of activation could be clearly shown.

Although the removal of the smear layer is recommended in the clinical setting, the irrigation protocol did not include EDTA due to its capacity to dissolve inorganic components of the smear layer and to damage the biofilm structure (Busanello et al., 2019). Since the aim was to analyse the effects of activation on biofilm removal and bacterial reduction, the additional use of EDTA would have added a confounding factor and might have masked differences between irrigation protocols.

## CONCLUSIONS

In a laboratory model using extracted human maxillary anterior teeth, high-frequency sonic activation resulted in greater bacterial reduction compared to ultrasonic activation in groups receiving solely irrigation/activation protocols. Both activation methods revealed greater bacterial reductions compared to nonactivated irrigation and highlight the importance of irrigant activation even in straight root canals.

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## ETHICS STATEMENT

This study was previously approved by the Ethical Committee of the Charité – Universitätsmedizin Berlin (protocol number EA4/102/14).

## CONFLICT OF INTERESTS

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

## AUTHOR CONTRIBUTION

Statistical analysis and interpretation of the data, writing the manuscript: Daniela Hoedke. Performing laboratory experiments, reviewing the manuscript: Namira Kaulika. Editing of the manuscript: Henrik Dommisch. Performing Fluorescence in vitro fluorescence, editing of the manuscript: Sebastian Schlafer. Editing the manuscript: Hagay Shemes. Development study design, interpretation of the data, writing the manuscript: Kerstin Bitter.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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