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Effect of photodynamic therapy in combination with various irrigation protocols on an endodontic multispecies biofilm *ex vivo*

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Abstract

Hoedke D, Enseleit C, Gruner D, Dommisch H, Schlafer S, Dige I, Bitter K. Effect of photodynamic therapy in combination with various irrigation protocols on an endodontic multispecies biofilm *ex vivo*. *International Endodontic Journal*.

Aim To analyse the antibacterial effect of photodynamic therapy (PDT) in combination with various irrigation protocols on a multispecies biofilm in root canals *ex vivo*.

Methodology A total of 160 extracted human single-rooted teeth were divided into four groups ($n = 40$). In group G1, root canals were instrumented up to size 60 (control group), whereas in G2 to G4 canals were enlarged up to size 40. All root canals were inoculated with a multispecies biofilm (*Enterococcus faecalis*, *Streptococcus oralis*, *Prevotella intermedia*) for 5 days. In G2 to G4, instrumentation up to size 60 was performed using 0.9% sodium chloride (NaCl) (G2), 1% sodium hypochlorite (NaOCl) (G3), 1% NaOCl and a final irrigation with 2% chlorhexidine (CHX) (G4), respectively. In all groups half of the specimens received adjunctive PDT using phenothiazine chloride as photosensitizer and a diode laser (wavelength 660 nm). Counts of colony-forming units (CFUs) in each group were analysed separately for planktonic and dentine-adherent bacteria immediately after therapy (T1) and after 5 days of further incubation (T2). Descriptive statistics and two-way analysis of variance were carried out to analyse reduction of planktonic bacteria and nonparametric tests were used to analyse dentine-adherent bacteria.

Results CFU reduction in planktonic bacteria was significantly affected by the irrigation protocol at T1 and T2 ($P < 0.0001$), but PDT significantly reduced CFUs only at T2 ($P = 0.01$; ANOVA). Irrigation using NaOCl, CHX and adjunctive PDT significantly reduced CFUs at T2 ($P < 0.0001$; Tukey HSD) compared to the control group. In 85.6% of all samples the same categories of CFU counts in both planktonic and dentine-adherent bacteria were detected at T1 and T2.

Conclusions Adjunctive photodynamic therapy in combination with an irrigation protocol including NaOCl and CHX was an effective method for reduction of bacterial biofilm inside the root canals of extracted teeth.

Keywords: antibacterial photodynamic therapy, irrigation protocol, multispecies biofilm, root canal disinfection.

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Introduction

Apical periodontitis is an inflammatory disease that is strongly correlated with microbial infection of the root canal system (Siqueira & Rôças 2014). To control the endodontic infection, the following elements have to

be considered: host defence, instrumentation and irrigation of the root canal system, intracanal medicaments between appointments, root filling and coronal restoration (Haapasalo & Shen 2012). Nearly 500 microbial species have been detected in endodontic infections. Phyla with the highest species richness were *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* (Siqueira & Rôças 2009). *Enterococcus faecalis* (*E. faecalis*) has been described as the most frequent species found in retreatment cases with a prevalence of up to 90%; whilst other less frequent bacteria, such as *streptococci*, were also found in retreatment cases (Rôças *et al.* 2004, Siqueira & Rôças 2004). Bacteria in the root canal system grow mostly in sessile biofilms; consequently the success of root canal treatment depends on effective elimination of such biofilms and removal of vital or necrotic tissues from the root canal system, which is considered the main goal of root canal treatment. Irrigating solutions should exhibit a strong antimicrobial effect, inactivate endotoxins, remove remnants of both pulp tissue and debris and dissolve both organic as well as inorganic material (Zehnder 2006). Sodium hypochlorite (NaOCl) solutions are the most frequently used irrigants because of their broad antimicrobial spectrum and their capacity to dissolve organic tissue remnants (Haapasalo & Shen 2012). Final irrigation of the root canal system using chlorhexidine in addition to NaOCl during instrumentation is frequently discussed in the literature, and this appears to be advantageous in retreatment cases due to its substantivity and antimicrobial effect against Gram-positive bacteria (Zehnder 2006, Haapasalo & Shen 2012, Cachovan *et al.* 2013). Although chemomechanical debridement of the root canal is quite effective, complete disinfection of inaccessible areas is sometimes difficult to achieve and remains challenging due to complex anatomy and biofilm resistance (Shen *et al.* 2012).

Antibacterial photodynamic therapy (PDT) is a two-step procedure including the application of a photosensitizer (step 1: photosensitization of infected tissue) that is followed by light illumination of this tissue (step 2: irradiation of the photosensitized tissue) resulting in toxic photochemistry and cell lysis (Kishen 2012). The activation of the photosensitizer occurs through light exposure with a specific corresponding wavelength in the presence of oxygen and generates singlet oxygen and radical ions leading to lethal damage of the bacterial cells, for example DNA damage and cytoplasmic membrane damage (Konopka & Goslinski 2007, Gursoy *et al.* 2013). Phenothiazinium photosensitizers such as methylene blue and toluidine blue are generally accepted and widely applied (Wainwright & Crossley 2002). For methylene blue-mediated PDT on *E. faecalis* cells, functional impairment of the cell wall, extensive damage of chromosomal DNA and degradation of membrane proteins have been reported (George & Kishen 2008).

In addition to chemomechanical debridement of the root canal for teeth with apical periodontitis, PDT has been suggested as a promising adjunct therapy in single-visit root canal treatment (Silva *et al.* 2012). Clinical data with a limited number of patients suggested a positive effect of adjunctive PDT on bacterial reduction in primary root canal treatment (Garcez *et al.* 2008) as well as in root canals harbouring multidrug resistant bacteria (Garcez *et al.* 2010). However, these studies did not correlate their results to clinical outcomes. Recent *ex vivo* data also demonstrated an enhanced antimicrobial effect of adjunctive PDT in combination with antibacterial irrigation of *E. faecalis* biofilms inside the root canal (Rios *et al.* 2011, Tennert *et al.* 2014, 2015). A recent review, however, questioned the efficacy of PDT on effective elimination of *E. faecalis* (Siddiqui *et al.* 2013). Therefore, the aim of the present study was to evaluate the effect of PDT on bacterial reduction of a multispecies biofilm in root canals in combination with different irrigation protocols. Moreover, the long-term antimicrobial effect of the various disinfection methods after further incubation was determined. The null hypothesis was that both irrigation protocols and adjunctive PDT have no effect on bacterial reduction immediately after treatment as well as after 5 days of further incubation.

Materials and methods

Sample preparation

A total of 160 extracted intact human single-rooted teeth with a single canal without distinct curvature 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 cleaned with ultrasonic scalers (SONICFlex; KaVo, Biberach, Germany). Crowns were removed, all roots were shortened to 19 mm, and all samples were sterilized by plasma sterilization (Campus Benjamin Franklin, Charité - Universitätsmedizin Berlin, Berlin, Germany).

Subsequently, all teeth were randomly divided into four main groups (n = 40). The coronal portion of the root canals was enlarged using Gates Glidden burs size 6 to 4. In group G1, apical root canal enlargement was performed up to size 60 with .02 taper, whereas instrumentation limited to size 40, .02 taper was carried out in G2 to G4 using the Flexmaster system (VDW, Munich, Germany). Irrigation was performed using sterile sodium chloride (NaCl, 0.9%, pharmacy of Charité – Universitätsmedizin Berlin, Germany). After initial root canal instrumentation, the smear layer was removed in all samples using ethylenediamine-tetraacetic acid 18% (Ultradent® EDTA 18%, Ultradent Products USA, Cologne, Germany). After covering the root surfaces with nail varnish (Long Lasting Nail Colour; Rival de Loop, Berlin, Germany), each tooth was embedded into closable cryo-tubes (Carl Roth, Karlsruhe, Germany) using epoxy resin (Technovit 4071; Heraeus Kulzer, Hanau, Germany). Subsequently, all teeth were plasma sterilized once again. Prior to bacterial inoculation sterility was tested by storing the teeth in sterile boxes (50 mL Falcon tubes; Sarstedt, Numbrecht, Germany) with sterile brain-heart media (BHI; SIFRIN, Berlin, Germany) at 37 °C under anaerobic conditions for 5 days. Clear media after 7 days indicated sterility. The study design is illustrated in Fig. 1.

Bacterial inoculation

Following sterilization, the root canals were inoculated with bacterial suspensions of *E. faecalis*, *Streptococcus oralis* (*S. oralis*) and *Prevotella intermedia* (*P. intermedia*), which were isolated from one infected root canal. Identification of the bacteria was performed by enzymatic reactions using enzymatic reaction test kits (API®, bioMérieux; Durham, North Carolina, USA). Prior to inoculation the bacterial species were grown on culture plates (Columbia agar plates with 5% sheep blood; Heipha, Eppelheim, Germany) for 3 days at 37 °C under anaerobic conditions using 2.5 L anaerobic jars (Anaerojar, Oxoid

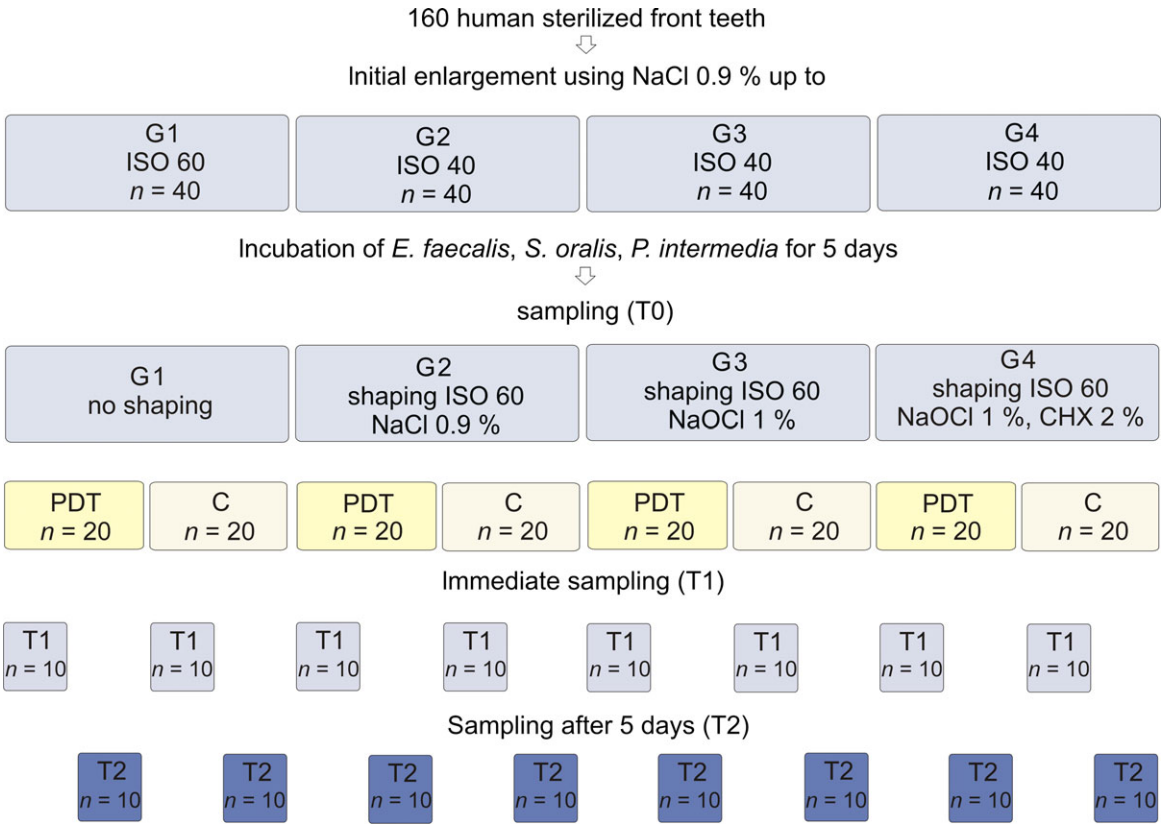

Figure 1 Study design visualized by flow chart. Sample preparation, treatment procedure, and sampling of planktonic and adherent bacteria are illustrated. G, group; NaCl, sodium chloride; NaOCl, sodium hypochlorite; CHX, chlorhexidine; C, control; PDT, photodynamic therapy; T, time.

Ltd, Basingstoke, UK) together with an appropriate anaerobic gas kit (AnaerobGen™ 2.5L, Oxoid Ltd) providing gas concentrations of <0.1% O_2, and 7–15% CO_2 according to the manufacturer's information). Bacteria were suspended in BHI separately and adjusted at a wavelength of 600 nm (Novaspec II Visible Spectrophotometer; Amersham Pharmacia Biotech AB, Uppsala, Sweden) to an optical density of 0.25 for *E. faecalis*, 1.0 for *S. oralis* and 1.0 for *P. intermedia* spectrophotometrically according to preliminary testing results that demonstrated adequate amounts of all three bacteria after applying different optical densities. A mixed media of the three bacterial species in the same proportions was placed into the root canal using a sterile needle and syringe (5 mL Syringe; BD Plastipak™, Franklin Lakes, New Jersey, USA) until the root canals were completely filled with bacterial suspension. Intracanal biofilms were grown for 4 days under anaerobic conditions at 37 °C with daily adding of 5 μL BHI to ensure constant medium supply in the root canals. Preliminary testing according to growth series of all bacteria revealed adequate growing of planktonic bacteria after 4 days of incubation (*E. faecalis* 8.0×10^8 CFUs mL^{-1}, *S. oralis* 6.4×10^8 CFUs mL^{-1}, *P. intermedia* 17.2×10^8 CFUs mL^{-1}).

Root canal treatment

In G2 to G4 root canal enlargement up to size 60, .02 taper was performed using sterile 0.9% NaCl in G2 and using 1% NaOCl (pharmacy of Charité - Universitätsmedizin Berlin, Germany) in G3 and G4. In G4, an intermediate rinse with ethanol (2-Propanol 70%, Emprove® exp; Merck KGaA, Darmstadt, Germany) was followed by final irrigation with 2% CHX (pharmacy of Charité - Universitätsmedizin Berlin, Germany). During instrumentation, irrigation was performed using 2 mL solution after each change of file size and final irrigation using 3 mL solution in each group. In G4, final irrigation was carried out using 3 mL NaOCl, followed by 3 mL ethanol and 3 mL CHX. Finally, root canals were irrigated with NaCl containing 3% Tween 80 (Tween 80, Sigma-Aldrich Chemie GmbH, Munich, Germany) and 0.3% L-α-lecithin (L-alpha-Lecithin, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for neutralizing CHX to avoid any carry-over effects of CHX on the agar plates. All root canals were dried using size 60 paper points (VDW, Munich, Germany).

After shaping and irrigation, each group was divided into two subgroups ($n = 20$). Subgroup 1 served as a control group and received no further treatment. In subgroup 2, PDT was performed using 10 mg mL^{-1} phenothiazin-5-ium,3,7-bis(dimethylamino)-,chloride (Helbo Endo Blue, Helbo, Grieskirchen, Austria) as photosensitizer and a diode laser with a wavelength of 660 nm and a power of 100 mW m^{-2} (Helbo Theralite Laser, Helbo, Grieskirchen, Austria). The photosensitizer was placed into the root canals for 60 s, and then rinsed out using 5 mL sterile 0.9% NaCl. Subsequently, the root canals were dried with sterile size 60 paper points. The optical tip of the laser (Helbo 3D Endo Probe, Helbo) with a diameter of approximately 0.45 mm and a length of the active surface of 17 mm was placed completely into the root canal and the laser was activated for 60 s. Energy fluency from the optical tip is >40 mW according to the manufacturer's information, so a total energy deposit of approximately 2.4 J was applied into each root canal. After treatment, all root canals were filled with sterile 0.9% NaCl.

Sampling of planktonic and adherent bacteria and determination of CFUs

Sampling of bacteria was performed at three different time-points: before treatment (T0) ($n = 160$), immediately after therapy (T1) ($n = 80$), or after 5 days of incubation under anaerobic conditions at 37 °C following therapy (T2) ($n = 80$). Sampling of planktonic bacteria from each canal was performed by inserting one sterile size 40 paper point (VDW, Munich, Germany) completely into the root canal until it was soaked with liquid. Each paper point was placed into 1995 μL BHI and vortexed for 30 s.

Bacteria from dentine were recovered by moving a size 40 Hedström file three times along the dentine wall from apical to coronal, placing the file into 50 μL BHI in a cryo-tube and vortexing for 30 s. At T0, dentine samples were taken from three additional root canals to verify baseline infection. At T1 and T2, dentine samples were taken after sampling planktonic bacteria using paper points.

Bacterial suspensions of each sample were diluted serially before plating on culture plates. All plates were incubated under anaerobic conditions for 5 days at 37 °C. The number of colony-forming units (CFUs) per mL was determined on three replicate plates per sample.

Sample preparation for Fluorescence-in-situ-Hybridization (FISH)

To validate the infection technique four inoculated roots with the above described multispecies biofilm

were fixed in 4% paraformaldehyde (3 vols.) in PBS (1 vol.) for 16 h at 4 °C, then washed with sterile PBS and stored in a mixture of 100% ethanol and PBS (1 : 1) at −20 °C. Thereafter, the roots were embedded in cold polymerizing resin (Technovit 8100; Heraeus Kulzer, Hanau, Germany) at 4 °C, as previously described (Zijnge *et al.* 2010, Dige *et al.* 2014). Subsequently, for each root, a 1 mm slice was cut parallel to the root canal with a saw microtome (Ernst Leitz GmbH, Wetzlar, Germany) and then decalcified in 17% ethylenediamidetetraacetic acid for 16–27 days. Digital X-ray analysis was performed to confirm complete decalcification. The root slices were sectioned into five blocks, the first from the most coronal part of the canal and the last from the apical part of the canal. All blocks included part of the root canal with the biofilm and the adjacent dentine. The slices were then re-embedded in cold polymerizing resin (Technovit 8100). Using a glass knife in an ultramicrotome (Ultracut E; Reichert Jung Optische Werke AG, Wien, Austria), 1–2 μm thin sections were cut, stretched on water and mounted on polysine coated glass slides (Menzel-Gläser, Braunschweig, Germany) prior to FISH analysis.

Fish

Probes STR405 (5′-TAG CCG TCC CTT TCT GGT-3′) (Paster *et al.* 1998), PRIN (5′-CTT TAC TCC CCA ACA AAA GCA GTT TAC AA-3′) (Sunde *et al.* 2003) and EUB 338 (5′-GCT GCC TCC CGT AGG AGT-3′) (Amann *et al.* 1990) were employed. STR405, targeting *streptococci*, was 5′-end-labelled with Alexa488 (MWG Biotech, Ebersberg, Germany), PRIN, targeting *P. intermedia*, was 5′-end-labelled with Atto550 (IBA, Göttingen, Germany), and the broad-range bacterial probe EUB 338 was 5′-end-labelled with Atto633 (IBA). FISH was performed as described previously (Manz *et al.* 1992, Dige *et al.* 2009). All specimens were treated with 25 μL lysozyme (Sigma, Brøndby, Denmark) (70 U μL^{-1} in 100 mmol L^{-1} Tris/HCl, pH 7.5, 5 mmol L^{-1} EDTA) for 9 min at 37 °C in a humid chamber prior to the FISH procedure for permeabilization. Specificity of STR405 was assessed with *S. oralis* as positive control and *P. intermedia* and *E. faecalis* (DSM 20478) as negative controls. *P. intermedia* was used as positive control for PRIN, and *S. oralis* and *E. faecalis* as negative controls. Hybridizations of root specimens were performed at 30% formamide. Prior to microscopy, a cover glass with Citifluor AF1 (Citifluor Ltd, Leicester, UK) was mounted.

Microscopy

A Zeiss LSM 700 Confocal Laser Scanning microscope (CLSM) (Carl Zeiss, Jena, Germany) was used for visualization of the biofilms. Alexa488, Atto550 and Atto633 were excited with 488, 543 and 639 nm laser lines, respectively. A 405 nm laser line was used to excite autofluorescence from dentine. A 100×/1.46 NA oil immersion objective (alpha Plan-Apochromat, Zeiss) was used for image acquisition. Images were 584 × 584 pixels (64 × 64 μm^2) in size and were acquired with pixel dwell time 5.53 μs, line average 2, zoom 1 (0.11 μm $pixel^{-1}$).

Statistical analysis

For statistical analysis, CFUs of all three bacteria from each sample were summed to define the CFUs of the biofilm. Additionally, CFUs of the planktonic as well as the adherent cells at T1 and T2 were categorized into three categories: no CFU, less than 100 000 CFUs per mL and more than 100 000 CFUs per mL. Descriptive analyses for metric and categorical data were performed. Statistical analysis was stratified by sampling time (T1 immediately after therapy, T2 after five additional days of incubation) and the location of the bacteria (planktonic bacteria of the root canal lumen sampled by paper points, or bacteria adhering to dentine walls sampled by Hedström files).

Before and after therapy, CFU counts of the planktonic bacteria were transformed in *log* 10 scale and logarithmic reduction factors were calculated. One-way ANOVA was performed for comparison of baseline infection. Two-way analysis of variance using logarithmic reduction factor as dependent variable were carried out to determine the effect of irrigation protocol (factor 1) and of adjunctive photodynamic therapy (factor 2). *Post hoc* tests (Tukey HSD) were performed to assess differences in the effects of different irrigation protocols and *t*-tests were calculated to analyse adjunctive PDT effects within the same group.

Kruskal–Wallis tests were performed to compare the distribution of categories of all groups of adherent bacteria after therapy. Mann–Whitney *U* tests stratified for PDT were carried out for pairwise comparisons using G1 (no shaping) as reference group. Regarding multiple testing in Mann–Whitney *U* tests alpha <0.0167 was considered to be statistical significant according to Bonferroni correction. Additionally, samples with positive culture after therapy were compared.

All analyses were performed using IBM SPSS statistics 22 (SPSS, IBM, Munich, Germany).

Results

Planktonic and dentine-adherent bacteria

With respect to the relative distribution of planktonic bacterial species 12 of 16 groups demonstrated *E. faecalis* as the most abundant bacterial species (range 12.9 – 68.6%) at T1 and T2. In ten of 16 groups, *P. intermedia* was the species with the lowest abundance (range 9.4 – 43.3%). The relative frequency of *S. oralis* ranged from 11.2% to 77.6% (Figure S1).

For planktonic bacteria, One-way ANOVA detected no differences for *logarithmic* transformed CFUs at T0 ($P = 0.83$). At T1 *logarithmic* bacterial reduction was significantly affected by the applied irrigation solution ($P < 0.0001$) but not by PDT ($P = 0.48$), and a significant interaction between the two factors could be observed ($P = 0.01$; ANOVA). Concerning the irrigation protocol, G3 and G4 demonstrated significant higher bacterial reduction compared to G1 ($P < 0.0001$; Tukey HSD), whereas G1 and G2 did not differ ($P = 0.091$; Tukey HSD). Significantly higher bacterial reductions were detected after PDT in G1 ($P = 0.026$; *t*-test) and G2 ($P = 0.01$; *t*-test) but not in G3 and G4. Median remaining amounts of bacteria were 3.1×10^6 ($0.9–52.7 \times 10^7$) CFUs per mL in G1 with PDT compared to 1.0×10^8 ($0.6–1.7 \times 10^9$) CFUs per mL in G1 without PDT and 1.4×10^6 ($0.7–15.3 \times 10^6$) CFUs per mL in G2 with PDT compared to 2.7×10^7 ($0.4–5.2 \times 10^7$) CFUs per mL in G2 without PDT (Table 1).

After 5 days (T2), bacterial reduction was significantly affected by the irrigation solution ($P < 0.0001$), PDT ($P = 0.01$) and the interaction between both factors ($P < 0.0001$; ANOVA). G4 revealed significantly higher bacterial reduction compared to G1 ($P < 0.0001$; Tukey HSD), whereas G2 and G3 did not differ significantly from G1 ($P > 0.05$; Tukey HSD). Significantly higher bacterial reductions were detected after PDT in G4 ($P = 0.001$; *t*-test). Median bacterial counts in G4 with PDT after 5 days were 2.3×10^3 ($0.8–24.9 \times 10^3$) CFUs per mL compared to 1.7×10^7 ($0.4–8.7 \times 10^7$) CFUs per mL in G4 without PDT (Fig. 2).

All three dentine samples collected to assess baseline infection at T0 harboured more than 100 000 CFUs mL^{-1} showing all three bacterial species. In G1 without PDT (control group) more than

Table 1 Description of CFUs per mL from the root canal lumen at baseline (T0), immediately after therapy (T1) and, after 5 days (T2)

Group	G1 (No shaping)		G2 (Shaping with saline)		G3 (Shaping with 1% NaOCl)		G4 (Shaping with 1% NaOCl and 2% CHX)	
Sub group	Control	PDT	Control	PDT	Control	PDT	Control	PDT
CFUs per mL T0	9.0×10^9 ($3.0–16.9 \times 10^9$)	2.2×10^9 ($0.8–5.1 \times 10^9$)	4.3×10^9 ($1.8–14.1 \times 10^9$)	3.8×10^9 ($3.0–7.4 \times 10^9$)	6.3×10^9 ($0.9–14.0 \times 10^9$)	7.0×10^9 ($3.2–14.8 \times 10^9$)	4.7×10^9 ($2.8–16.3 \times 10^9$)	5.2×10^9 ($2.0–8.8 \times 10^9$)
CFUs per mL T1	1.0×10^8 ($0.6–1.7 \times 10^9$)	3.1×10^6 ($0.9–52.7 \times 10^7$	2.7×10^7 ($0.4–5.2 \times 10^7$)	1.4×10^6 ($0.7–15.3 \times 10^6$)	7.5×10^2 ($7.5–7.5 \times 10^2$)	1.4×10^3 ($0.6–140 \times 10^3$)	1.61 × 103 ($0.8–5.7 \times 10^3$)	1.9×10^3 ($0.8–15.7 \times 10^3$)
Irf 1	1.9 ± 0.3	2.6 ± 0.8	2.4 ± 0.4	3.3 ± 0.9	6.7 ± 0.7	5.9 ± 1.6	6.4 ± 0.6	6.1 ± 0.9
CFUs per mL T0	5.2×10^9 ($1.8–10.7 \times 10^9$)	4.8×10^9 ($2.9–11.5 \times 10^9$)	5.7×10^9 ($3.2–11.1 \times 10^9$)	3.8×10^9 ($2.1–9.9 \times 10^9$)	5.0×10^9 ($1.9–9.7 \times 10^9$)	6.2×10^9 ($1.6–11.0 \times 10^9$)	5.7×10^9 ($2.4–10.0 \times 10^9$)	2.9×10^9 ($1.8–8.6 \times 10^9$)
CFUs per mL T2	0.8×10^8 ($0.3–2.9 \times 10^9$)	2.4×10^9 ($1.2–5.8 \times 10^9$)	0.9×10^9 ($0.4–2.9 \times 10^9$)	1.4×10^9 ($0.6–5.2 \times 10^9$)	6.2×10^9 ($5.9–13.1 \times 10^9$)	5.9×10^9 ($0.4–17.4 \times 10^9$)	1.7×10^7 ($0.4–8.7 \times 10^7$)	2.3×10^3 ($0.8–24.9 \times 10^3$)
Irf 2	0.8 ± 0.5	0.3 ± 0.6	0.4 ± 0.5	0.4 ± 0.5	−0.13 ± 0.6	0.3 ± 1.1	3.0 ± 1.8	5.7 ± 1.4

Data are median (interquartile range) or mean ± standard deviation.
CFUs, colony-forming units; PDT, photodynamic therapy; NaOCl, sodium hypochlorite; CHX, chlorhexidine; Irf, logarithmic reduction factor.

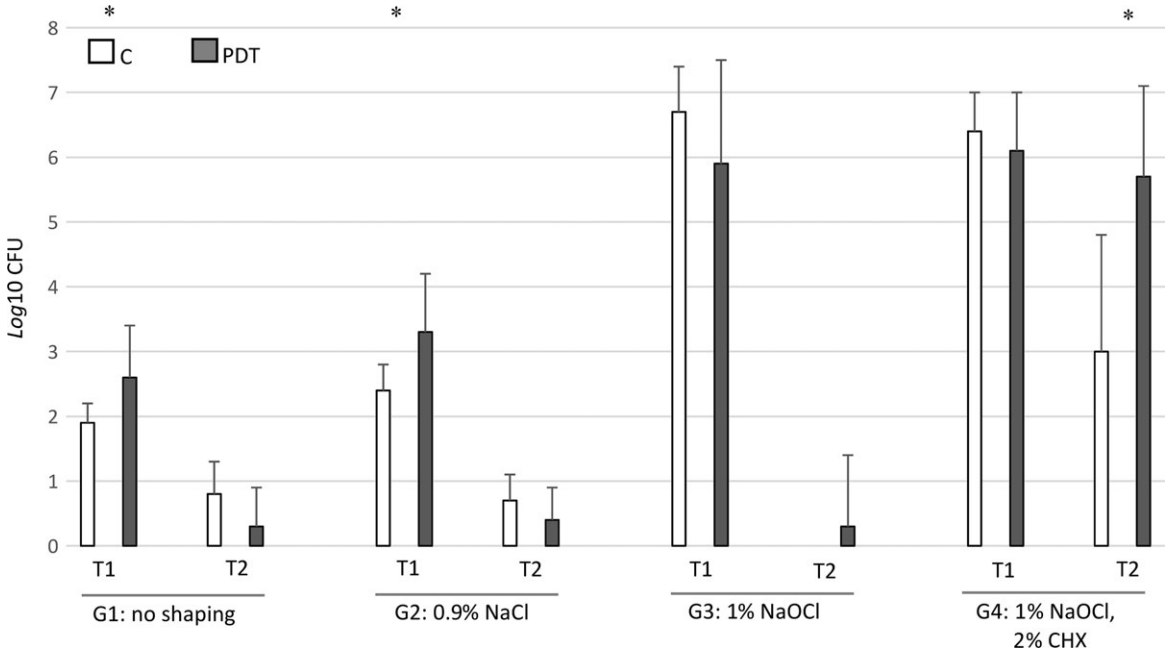

Figure 2 Logarithmic reduction of planktonic bacteria for treatment groups and subgroups at sampling times T1 and T2. Bars represent mean logarithmic CFU reduction of planktonic bacteria stratified for treatment groups (G1–4), subgroups (control (C) and photodynamic therapy (P)), and sampling time-point (immediate sampling (T1), sampling after 5 days (T2)). ∗At T1 significantly higher bacterial reductions were detected after PDT in G1 ($P = 0.026$) and G2 ($P = 0.01$; *t*-test). At T2 in G4 (shaping with NaOCl and CHX) CFU reduction was significantly higher with PDT compared to control ($P = 0.004$, *t*-test). NaCl, sodium chloride; NaOCl, sodium hypochlorite; CHX, chlorhexidine; log, logarithmic; CFUs, colony-forming units.

100 000 CFUs mL^{-1} of all three bacterial species were detected in 80% of all dentine samples.

The Kruskal–Wallis test detected significant differences in categories of CFUs between groups at T1 ($P < 0.0001$) as well as at T2 ($P < 0.0001$). G4 revealed significant differences compared to G1 at T1 with and without PDT ($P < 0.0001$) and at T2 solely with PDT ($P \leq 0.0001$; Mann–Whitney *U* test) as indicated in Fig. 3. The number of samples with positive culture differed significantly between the main groups at T1 with and without PDT ($P = 0.001$; Kruskal–Wallis test) and at T2 only in groups with application of PDT ($P < 0.001$, Kruskal–Wallis test).

Comparison of planktonic and adherent bacteria

Descriptive data revealed that after treatment (T1 and T2) in 26 (16.3%) of 160 teeth no planktonic bacteria could be detected. Furthermore, in 23 teeth (14.4%) up to 100 000 CFUs mL^{-1}, and in 111 teeth (69.4%) more than 100 000 CFUs mL^{-1} deriving from the planktonic samples were counted. Adherent bacteria could not be detected in 35 root canals (21.9%); in 19 (11.9%) samples up to 100 000 CFUs mL^{-1}, and in 106 (66.3%) samples more than 100 000 CFUs mL^{-1} were detected.

In 137 samples (85.6%) the same categories of CFUs (no CFUs, less than 100 000 CFUs mL^{-1} or more than 100 000 CFUs mL^{-1}) were found in both paper point and dentine samples (Kappa = 0.71). In G3 and G4, 26 root canals without planktonic bacteria were observed. In 22 of the 26 teeth without CFUs from paper point samples, no CFU could be detected in dentine samples, either.

Fish

At 30% formamide, *P. intermedia* was reliably targeted by probes PRIN and EUB338, but not by STR405 (Figure S2a–c). *S. oralis* was detected by STR405 and EUB338, but not by PRIN (Figure S2d–f). Confocal microscopy analysis of sectioned tooth specimens showed a thin biofilm covering both coronal and apical areas of the root canal system. *P. intermedia* and *S. oralis*, visualized with specific probes, and *E. faecalis*, visualized by EUB338 only, were identified in close spatial relation to each other (Fig. 4a,b). In some areas, cells of *S. oralis* and *E. faecalis* had invaded dentinal tubules (Fig. 4c).

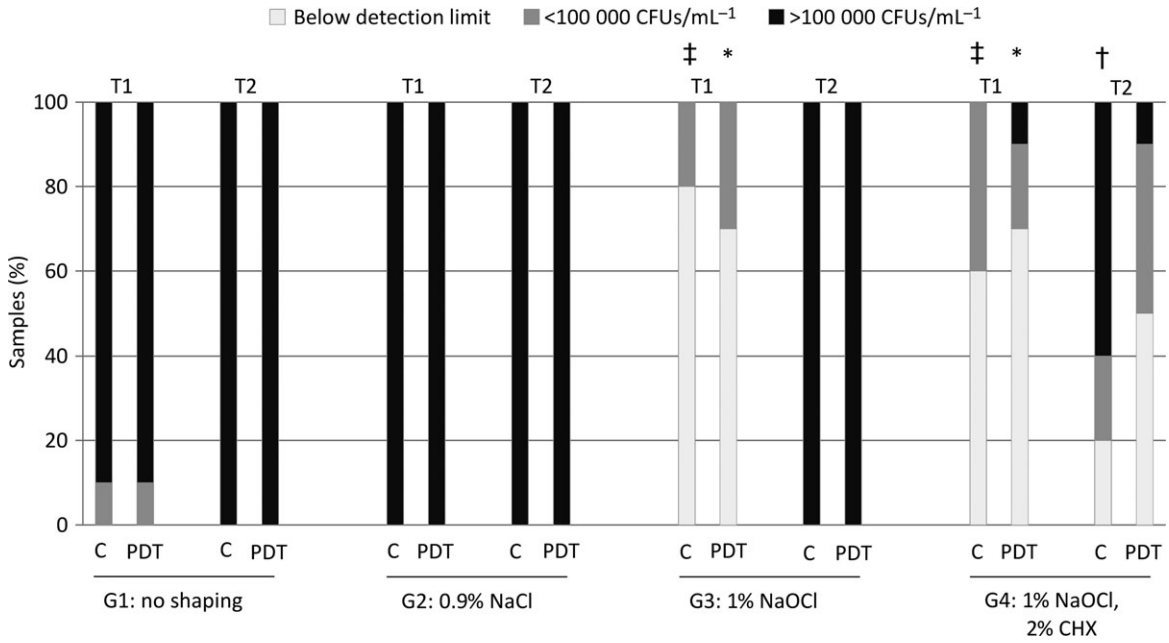

Figure 3 CFU categories from adherent bacteria after therapy. Numbers of samples within three categories (below detection limit, less than 100 000 CFUs mL^{-1} or more than 100 000 CFUs mL^{-1}) are stratified for treatment groups (G1–4), subgroups (PDT (P), control (C)), and sampling time-point (immediate sampling (T1), sampling after five more days of incubation (T2)). ∗G3 and G4 with PDT differed significantly from G1 with PDT at T1 ($P < 0.0001$, Mann-Whitney U tests). †G4 with PDT revealed significant differences from G1 with PDT at T2 ($P < 0.0001$, Mann-Whitney U test). ‡In subgroups without PDT G3 and G4 showed significant differences compared to G1 at T1 ($P < 0.0001$, Mann-Whitney U tests), NaCl, sodium chloride; NaOCl, sodium hypochlorite; CHX, chlorhexidine.

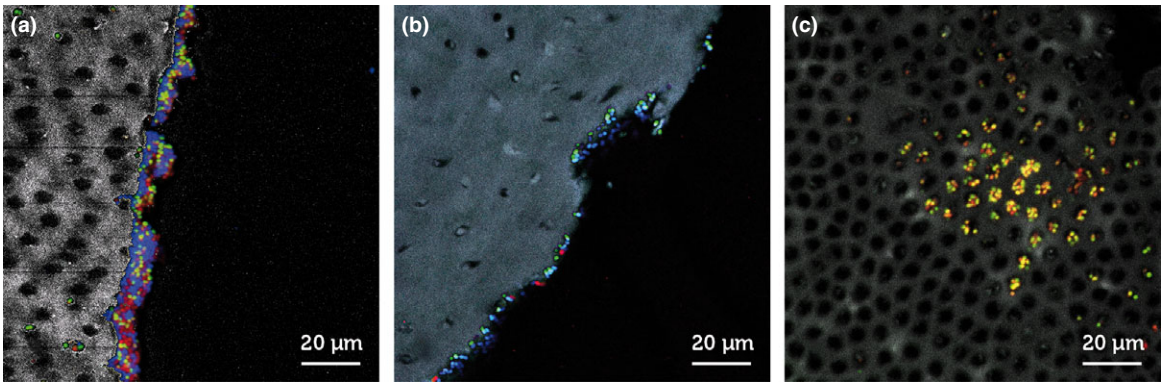

Figure 4 Biofilms in root canal sections visualized by FISH. The probes STR405 (displayed in green), specific for streptococci, PRIN (blue), specific for *Prevotella intermedia*, and EUB338 (red), targeting all bacteria, were employed simultaneously. Autofluorescence from dentine is shown in grey. A thin biofilm consisting of *Streptococcus oralis*, *P. intermedia* and *Enterococcus faecalis* (targeted by EUB338 only) is shown firmly attached to the dentine wall in coronal (a) and more apical parts (b) of the root canal. In some areas, cells of *S. oralis* and *E. faecalis* invade dentinal tubules (c).

Discussion

The present study analysed several irrigation protocols as well as the application of PDT on a multispecies biofilm model of endodontic infection simulating a one-visit root canal treatment of retreatment cases. The null hypothesis of the study has to be partly rejected because the irrigation protocols

affected bacterial reduction immediately after treatment and after 5 days of further incubation for planktonic and adherent bacteria. Immediately after treatment chemomechanical debridement using 1% NaOCl or 1% NaOCl and 2% CHX were significantly more effective compared to the control group. After 5 days of further incubation, chemomechanical debridement using 1% NaOCl and 2% CHX revealed significantly lower bacterial load compared to the control group, whereas solely using 1% NaOCl did not differ significantly from the control group. A significant effect of adjunctive PDT on bacterial reduction could be demonstrated after 5 days of additional incubation and chemomechanical debridement using 1% NaOCl and 2% CHX. Effects of PDT in G1 and G2 at T1 on bacterial reduction were only 2-*log* reduction whereas the above mentioned effect was 6-*log* reduction.

Laboratory studies investigating antimicrobial effects of different disinfection methods should use models that closely resemble *in vivo* conditions (Shen *et al.* 2012). At the same time, reproducible infection and recovery of bacterial cells are important. Consequently, a multispecies biofilm model was employed with strains from an infected root canal of a patient with apical periodontitis that mimics the clinical situation better than a monospecies biofilm. Moreover, anterior teeth with canals instrumented up to size 40 with straight root canals were used to achieve a comparable bacterial load in all groups, to reduce anatomical variation and to allow standardized sampling. However, these enlarged root canals mimic the situation of young permanent teeth and, in combination with the bacterial strains retreatment cases.

The presence of all three organisms in the root canals before treatment was ascertained by paper point sampling after 2 and 4 days (T0) of incubation. To not disrupt the biofilm prior to experimental treatment, dentine samples were collected from three representative teeth at T0. Whilst it cannot be guaranteed that three-species biofilms were present in all teeth, representative dentine samples taken at T0 confirmed the attachment of all strains to the canal wall. Additionally, in the control group, where no additional instrumentation, irrigation or PDT was applied, all three bacterial species were detected in 80% of all dentine samples above the countable limit (<100 000 and >100 000 CFUs mL^{-1}). Moreover, successful biofilm formation in representative teeth was validated using FISH. It could be demonstrated that all three bacterial species were organized in a biofilm in close spatial relation to each other and that coronal as well as apical parts of the root canal were colonized (Fig. 4a,b). In some areas, cells of *S. oralis* and *E. faecalis* had invaded dentinal tubules, demonstrating that the biofilm was well established inside the canal (Fig. 4c). In the present study, *E. faecalis* was the predominant species inside the biofilms at both sampling times, as determined by CFU counts (Figure S1). The results indicate that the multispecies biofilm was relatively stable in its species composition, irrespective of the employed disinfection strategies.

Determination of CFUs inside the root canal has been regarded as the gold standard for evaluating the disinfection efficacy of various methods and has been applied in numerous studies *in vitro* (Shen *et al.* 2012). Whilst it is a cheap and easy method to analyse bacterial counts, it remains questionable whether CFU counts realistically reflect bacterial growth inside the root canal. On the one hand, organisms respond differently to removal from their habitat and subsequent culture on agar plates, and on the other hand, only part of the flora can be sampled with mechanical means. In the present study, sampling was performed with paper points to quantify planktonic bacteria and with files to collect bacteria adhering to the canal wall. Consequently, bacterial cells residing in areas inaccessible to mechanical debridement were not sampled.

In the present study, descriptive analyses of detected planktonic and adherent bacteria after the different treatment protocols suggest no distinctive differences between both sampling methods, although it should be assumed that the disinfecting strategies were more effective on planktonic bacteria. In most of the root canals without bacteria in the paper point samples no bacteria were detected in dentine samples. The calculated Kappa value (0.71) between both sampling methods revealed good agreement indicating that analyses of planktonic bacteria might allow conclusions about the antibacterial effects of disinfection strategies inside the root canal.

The present study analysed the bacterial counts immediately after treatment as well as after 5 days of further incubation simulating a worst-case scenario in unfilled areas of the root canal system. A recent study revealed that different bacteria survive in root canals after antimicrobial therapy for up to 5 days, and that the number of CFUs can increase 5 days after therapy (Cachovan *et al.* 2013). The present study also demonstrated a greater bacterial reduction immediately after therapy compared to 5 days of

further incubation. Apparently, remaining bacteria were able to proliferate within the 5 days of further incubation. Consequently, the results of T2 may be regarded as being more clinically relevant. NaOCl in concentrations of 1.0% and 5.0% have shown high antibacterial activity in a contact test (Sassone *et al.* 2003), and residual NaOCl inside dentinal tubules has been regarded as critical for effective disinfection (Hecker *et al.* 2013). Immediately after therapy, comparable effects on bacterial reduction of the irrigation protocols using solely 1% NaOCl or additional final irrigation using 2% CHX were observed.

Ethanol was used as an intermediate rinsing between NaOCl and CHX to avoid any precipitation (Krishnamurthy & Sudhakaran 2010). After 5 days of further incubation, the bacterial load was significantly lower for NaOCl and CHX compared to solely using NaOCl, both in paper point and in dentine samples. This observation is corroborated by both an *in vitro* and an *in vivo* study (Paiva *et al.* 2012, Cachovan *et al.* 2013). However, ultrasonic agitation of NaOCl increases the effectiveness of the final rinse procedure especially in the apical third of the root canal (Paragliola *et al.* 2010) and was not included in the present investigation. Consequently, enhanced effects of NaOCl irrigation using ultrasonic agitation compared to the present results could be expected.

Only weak effects of PDT on bacterial reductions without further chemomechanical debridement of the root canal could be detected immediately after treatment (T1) in the present study (2-*log* reduction). Although significant bacterial reductions have been detected in G1 and G2 after PDT the amount of remaining bacteria were still high and clinically unsatisfactory. This is in correspondence with previous studies (Souza *et al.* 2010, Muhammad *et al.* 2014, Tennert *et al.* 2014, 2015), suggesting that PDT might be an effective adjunctive method in root canal disinfection, but not a standalone treatment. In the present study, a significant effect of adjunctive PDT up to an adequate level (6-*log* bacterial reduction) could only be detected after 5 days of further incubation, but this effect was observed in combination with the irrigation protocol using NaOCl and CHX. Consequently, final irrigation using CHX might have positively affected penetration or binding of the photosensitizer inside the root canal, and therefore increased the antibacterial effect of PDT. However; the combination of NaOCl, CHX and PDT did not result in complete eradication of the intracanal biofilm, which emphasizes the possible necessity of bactericidal inter-appointment dressings in certain cases.

In this study, application of the photosensitizer and laser was conducted according to the manufacturer's recommendations. In the literature, various combinations of photosensitizers and light sources as well as modifications of the photosensitizer and application modes have been evaluated, and this affected the antimicrobial effect of PDT (Souza *et al.* 2010, Nunes *et al.* 2011, Rios *et al.* 2011, Tennert *et al.* 2015). In this context, further research is clearly warranted to potentially increase the antimicrobial effect of PDT. Current research is also focused on increasing the antibiofilm efficacy of PDT by combining the photodynamic effects with bioactive micro- and nanoparticles (Pagonis *et al.* 2010, Shrestha & Kishen 2012) to achieve a wider spectrum of PDT. Consequently, it has to be considered that alterations of the application of PDT as well as modifications of the photosensitizer might have an impact on the present results.

Conclusion

The present study demonstrated a greater reduction of bacteria belonging to a planktonic and adherent bacterial multispecies biofilm inside the root canal after chemomechanical debridement using 1% NaOCl and 2% CHX, compared to the control using NaCl. The combination of 1% NaOCl and 2% CHX was significantly more effective compared to 1% NaOCl alone after 5 days of further incubation. Adjunctive PDT enhanced the effect of chemomechanical debridement after 5 days of further incubation. Within the limitation of this *ex vivo* study, the results suggest that PDT in combination with an irrigation protocol including NaOCl and CHX was an effective method for reducing bacteria inside the root canal, although this disinfection method was not able to provide complete eradication of all bacteria inside the root canal.

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Conflict of interest

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

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Distribution of *E. faecalis*, *S. oralis* and *P. intermedia* in the planktonic samples. The relative frequencies of species recovered from paper point samples are shown stratified for treatment groups (G1–4), subgroups (control (C) photodynamic therapy (P)), and sampling time-point (immediate sampling (T1), sampling after 5 days (T2). *E, Enterococcus*; *S, Streptococcus*; *P, Prevotella*; NaCl, sodium chloride; NaOCl, sodium hypochlorite; CHX, chlorhexidine.

Figure S2. Specificity of hybridization experiments. At a formamide concentration of 30%, *Streptococcus oralis* was targeted by STR405 (a) and EUB338 (b), but not by PRIN (c). *Prevotella intermedia* was targeted by PRIN (d) and EUB338 (e), but not by STR405 (f).