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Model system parameters influence the sodium hypochlorite susceptibility of endodontic biofilms

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Abstract

Aim To evaluate the influence of several model system parameters on the sodium hypochlorite (NaOCl) susceptibility of endodontic biofilms. Based on these findings, a relevant *in vitro* endodontic biofilm model is proposed.

Methodology *In vitro* biofilms were cultured, varying the following experimental model parameters: biofilm composition (monospecies *Enterococcus faecalis* and a multispecies biofilm including *E. faecalis*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Porphyromonas gingivalis*), incubation time (24h or 11 days), incubation atmosphere (aerobically or anaerobically), and biofilm substrate (polystyrene microtiter plate wells, hydroxyapatite or dentine). Biofilms were subjected to treatment with NaOCl (0.025%, 0.1%, 0.5%, 2.5%) for 1 minute, control groups included treatment with purified water. Biofilms were harvested and the number of surviving cells was determined by plate counting using general (monospecies biofilms) or selective (multispecies biofilms) media. A two-way ANOVA was conducted to explore the effect of the model parameters on biofilm eradication. Finally, the most physiologically relevant biofilm model (11-day-old multispecies biofilm grown anaerobically on dentin discs) was characterized by selective media plate counting, NaOCl susceptibility testing, scanning and transmission electron microscopy.

Results There was no difference in NaOCl eradication between the anaerobically and aerobically grown *E. faecalis* biofilms. One-day-old biofilms of *E. faecalis* were more susceptible to most tested NaOCl concentrations than 11-day-old biofilms ($P < 0.05$). When grown in a multispecies biofilm, *E. faecalis* was significantly less susceptible to NaOCl treatment than in a monospecies biofilm ($P < 0.05$). *E. faecalis* in a multispecies biofilm grown in a MTP was more susceptible to NaOCl (0.025% and 0.1%) than when grown on hydroxyapatite or dentine. No difference in biofilm NaOCl susceptibility was seen between hydroxyapatite and dentine. The multispecies biofilm proved to be a reproducible model with high NaOCl resistance, complex structure and organization.

Conclusion The parameters biofilm age, biofilm composition and substrate had a significant influence on the NaOCl susceptibility of *E. faecalis* biofilms. Older biofilms, multispecies biofilms and biofilms grown on dentine and hydroxyapatite had reduced NaOCl susceptibility. These findings emphasize the importance of selecting relevant parameters when designing a biofilm model system for the evaluation of antimicrobial treatments.

Introduction

It is well established that apical periodontitis is an infectious disease caused by micro-organisms mainly organised as intra-canal biofilms (Nair *et al.* 1990, Chávez de Paz 2007, Ricucci & Siqueira 2010). Micro-organisms in a tooth with an established canal infection occur as integrated multispecies communities, embedded within a self-produced extra-cellular matrix and attached to the root canal walls (Nair 1987, Siqueira & Rôças 2009, Ricucci & Siqueira 2010). Studies based on molecular microbial identification techniques have shown that the infected root canal harbours a highly diverse microbiota, both in primary as well as in secondary infections (Siqueira *et al.* 2011, 2016, Ozok *et al.* 2012). The specific composition of the canal microbiota is defined by factors such as oxygen tension, interspecies interactions and the nutrient availability.

The treatment of apical periodontitis relies on the eradication or substantial reduction of the canal infection, through a combined mechanical and chemical cleaning and disinfection of the root canal space. Although success rates for this treatment modality are high (Ng *et al.* 2008), treatment failures do occur. Because treatment failure is mainly due to persistent infection (Sjögren *et al.* 1997, Molander *et al.* 1998), better and more predictable root canal disinfection protocols are continuously researched. In order to evaluate such new root canal disinfection modalities, it is relevant and common practice to first test these in laboratory models, in order not to waste valuable resources in clinical trials. To this end, *in vitro* endodontic biofilm model systems are used.

Such an endodontic biofilm model system should mimic the clinical situation as closely as possible, whilst at the same time provide sufficient practical feasibility and reproducibility. The endodontic literature is plentiful with studies using laboratory biofilm models. A recent literature review presented an overview of these systems and discussed the various factors that constitute these models (Swimberghe *et al.* 2018) and revealed considerable variation in the model systems deployed throughout the literature. As a consequence of these differences in experimental model parameters, comparison of studies is difficult at best. In addition, it was noted that in the majority of the studies, relatively simple model systems, using experimental parameters which are poorly reflective of the *in vivo* situation were used. For example, in 86% of the included studies, a monospecies biofilm was cultured. Only few studies described a biofilm model system with more than one species (Chen *et al.* 2013, Ferrer-Luque *et al.* 2014, Christo *et al.* 2016, De Meyer *et al.* 2017). In these multispecies biofilm studies, SEM, CLSM or total cell counts based on culturing were the most frequently used biofilm quantification methods (Xie *et al.* 2012, Shrestha & Kishen 2014, Mistry *et al.* 2015, Niazi *et al.* 2015, Almaroof *et al.* 2017, Rosa *et al.* 2017). These methods provide data on the amount and/or structure of the biofilm in general, but do not provide information about the individual species. Detailed insights into the presence and abundance of

constituting species is of great importance for a better understanding of the reaction of polymicrobial biofilms to antimicrobial treatment alongside with an improvement in the likelihood of treatment success (Lopes *et al.* 2018).

On the other hand, the use of more relevant models is challenging since more realistic parameters such as selection of strictly anaerobic microbial species, a multispecies setup, and species-specific quantification techniques are more demanding in terms of time consumption, technical requirements of the laboratory, and implicate more complex working conditions. It remains a question whether the outcomes obtained in relatively simple, less relevant models can be confirmed in complex models that have more resemblance to the *in vivo* situation. Previous studies have shown the influence of parameters such as the culture medium (Seneviratne *et al.* 2013), dentine age and pretreatment (Ozdemir *et al.* 2010), biofilm incubation time (Shen *et al.* 2011, Stojicic *et al.* 2013) on the susceptibility of the biofilm, or compared mono- versus dual-species biofilms (Ozok *et al.* 2007). However, these studies mostly focus on one parameter and fail to study the parameter influence comprehensively. In addition, the influence of multispecies composition and oxygen presence on the susceptibility of endodontic biofilms has not been studied yet.

Therefore, the aim of this study was to investigate the influence of several critical parameters of the biofilm model system on the susceptibility of endodontic biofilms to NaOCl. The null hypothesis is that parameters, such as incubation time, oxygen presence, and biofilm composition have no influence on the NaOCl susceptibility of the biofilm. Further, based on the findings, a relevant multispecies biofilm model is proposed.

Materials and methods

Bacterial strains and culture conditions

Four bacterial species (Table 1), typical members of primary and/or secondary endodontic infections, were selected. The bacteria were cultured from a frozen stock. The strict anaerobic bacteria were streaked onto Columbia Blood Agar (CBA; Columbia Agar base (Lab M, Bury, UK) + 5% defibrinated sheep blood (Biotrading, Mijdrecht, The Netherlands)), and cultivated anaerobically (5% CO₂, 5% H₂, 90% N₂) in an anaerobic chamber (Bactronez; Sheldon manufacturing Inc., Cornelius, Oregon, USA) for 72 h at 37°C. *Enterococcus faecalis* was grown aerobically on Brain Heart Infusion (BHI; Lab M) agar for 24 h at 37°C. Liquid cultures of all anaerobic strains were grown anaerobically in BHI broth at 37°C until stationary phase. Liquid cultures of *E. faecalis* were grown aerobically overnight at 37°C on a shaking platform.

For each species, a selective medium was chosen or developed to quantify the individual species in a multispecies community. Each selective medium allowed growth of the target species while

inhibiting growth of the other species (Table 1). The composition of the selective media is provided as supplementary material (S1). *E. faecalis* was plated on BHIA and incubated aerobically for 24 h. The aerobic environment prevented growth of the strict anaerobic bacteria. For isolation and quantification of *Fusobacterium nucleatum* in multispecies biofilms, Crystal Violet-Erythromycin Agar (CVE) was used (Walker *et al.* 1979). *Prevotella intermedia* was grown on Schaedler Kanamycin/Vancomycin Agar with 5% Sheep Blood (SKV) (van Winkelhoff & de Graaff 1983). *Porphyromonas gingivalis* was isolated on *Porphyromonas Gingivalis* Agar (P. GING) (Hunt *et al.* 1986) supplemented with 6.4 µg/mL mupirocine. The latter was required since P. GING did not inhibit the growth of *E. faecalis*. Mupirocine was selected after antibiotic susceptibility screening. Susceptibility of the four species to 25 antibiotics was tested using the disc diffusion assay according to EUCAST guidelines (EUCAST 2020). To this end, antibiotic discs (Oxoid, Wesel, Germany) were placed on inoculated CBA, and inhibition zones were measured after incubation. In order to determine the concentration of the antibiotic required for inhibition, the minimum inhibitory concentration (MIC) of the selected antibiotic was defined using the broth microdilution method according to EUCAST guidelines (EUCAST 2000).

For all the selective media, the quantitative recovery of the target species was tested. Recovery was considered quantitative when no significant difference was observed between the number of CFU on the general medium (CBA) compared to the selective medium. Quantitative recovery of cells was evaluated in untreated and in NaOCl treated (see below) biofilms (data not shown).

Biofilm models

Microtitre plate

Biofilms were grown in PVC round-bottom 96-well plates (Thermo Fisher, Waltham, MA USA), according to previously published protocols (Vandeplassche *et al.* 2017). The optical density of the planktonic cultures was adjusted spectrophotometrically to a density of 5×10^7 CFU/mL. The growth medium for the biofilms was BHI (Lab M) supplemented with 2.5% lysed horse blood (Biotrading). For the single-species biofilms the inoculum was 2.5×10^6 cells. For the multispecies biofilms the inoculum was 2.5×10^6 cells/species for the anaerobes and 2.5×10^2 cells for *E. faecalis*. The inoculation of the multispecies biofilm occurred in two phases. *P. gingivalis*, *P. intermedia* and *F. nucleatum* constituted the starting culture, *E. faecalis* was added after 8 days. The timing and inoculum size for the addition of *E. faecalis* was based on preliminary experiments showing these conditions to result in optimal species balance without biofilm overgrowth by *E. faecalis*. Every two days, biofilm supernatant was removed, biofilms were rinsed with physiological saline solution (PS) and fresh medium was added. The biofilms were incubated at 37°C under aerobic or anaerobic conditions. Monospecies *E. faecalis* biofilms were incubated for 24 h or 11 days. Multispecies biofilms were incubated for 11 days.

Hydroxyapatite and dentine discs

The crowns of noncarious, extracted human molars were milled to a diameter of 7 mm. The use of extracted teeth had been approved by the Ghent University Ethics Board (EC 2018/0633). These cylinders were then cut below the enamel–dentine junction and above the pulp space using a rotating diamond saw (Isomet Saw; Buehler Ltd, Evanston, IL, USA) to obtain standardized dentine slices of 2 mm thickness. These were suspended in EDTA (Pulpdent EDTA Solution 17%; Pulpdent Corporation, Watertown, MA, USA) for 1 min before being sterilized by autoclaving (121 °C, 20 min). Hydroxyapatite (7mm diameter x 1.8 mm thick, Clarkson Chromatography Products Inc., South Williamsport, PA, USA) and dentine discs were incubated overnight in BHI broth at 4°C. They were then placed in a PVC flat-bottom 12-well MTP (Thermo Fisher). Suspensions of *P. gingivalis*, *P. intermedia* and *F. nucleatum* in concentrations identical to those used in 96-MTP, together with 2.5% lysed horse blood and filled to 2 mL with BHI. Every two days, the discs were rinsed with PS and placed in a new well with fresh medium. On day 8, *E. faecalis* was added in the same concentration as in the 96-MTP.

Biofilm set-ups

Six different biofilm set-ups were used, varying incubation atmosphere, incubation time, biofilm substrate and biofilm composition Table 2 shows an overview of these set-ups.

NaOCl treatment of the biofilms

Sodium hypochlorite (3% w/v; Denteck, Zoetermeer, The Netherlands) was diluted with milliQ water (MQ) to concentrations of 2.5, 0.5, 0.1, 0.025 %. Prior to treatment, the biofilms were rinsed with PS to remove planktonic cells and debris. One hundred µL of NaOCl or MQ was added to the biofilms grown in the 96-well MTP. After 1 minute, the NaOCl or MQ was replaced with 200 µL neutralizing broth (NB; D/E Neutralizing Broth, Lab M) to neutralize the activity of the disinfectant. After 5 minutes the NB was removed and the biofilm was rinsed with PS. Then 100 µL PS was added to each well. The biofilms grown on discs were first rinsed in PS and then placed in 300 µL NaOCl or MQ for 1 minute and transferred to a well with 600 µL NB. After 5 minutes the discs were rinsed in 600 µL PS and placed in an Eppendorf tube filled with 1 mL PS. The susceptibility to NaOCl of monospecies *E. faecalis* biofilms when grown under varying oxygen conditions (aerobically and anaerobically) for 24 h was evaluated initially. These biofilms were then subjected to the previously described NaOCl concentrations (2.5, 0.5, 0.1. and 0.025%). In the second series of experiments, monospecies *E. faecalis* biofilms were incubated for varying periods of time (24 h and 11 days) anaerobically, before being treated with different NaOCl concentrations (2.5, 0.5, 0.1. and 0.025%). In the next experiment, the growth of *E. faecalis* varied as a single culture or a multi-species biofilm comprised of *E. faecalis*, *F. nucleatum*, *P. gingivalis*, and *P. intermedia* which was incubated anaerobically for 11 days, and

subjected both types of biofilm to identical antimicrobial treatment with NaOCl as in the latter test. In the fourth experiment, the growth substrate was varied, whereby multispecies biofilms were grown for 11 days on MTP, hydroxyapatite or dentine discs before treatment. All experiments were carried out in at least biological triplicate, and for every experimental condition at least 3 technical replicates were included.

Biofilm quantification

After treatment, MTP-grown cells were harvested from the wells by two cycles of 5 min shaking at 700 rpm (Titramax 1000; Heidolph Instruments GmbH, Schwabach, Germany) and 5 min sonication (Branson 3570EMT; Branson Ultrasonic Corp., Danbury, CT, USA), resulting in 200 μ L suspension per biofilm (Vandeplassche *et al.* 2017). The disc-grown biofilm cells were removed by three cycles of vortexing (30 s) and sonication (30 s). These suspensions were serially diluted and 10 μ L of each dilution was plated on the appropriate general and/or selective media. After incubation, the colonies were counted and the number of colony-forming units (CFU) was calculated. The CFU count in the untreated controls was defined as 100% survival. For the treatment groups, the % survival to the control group was calculated.

Statistical analysis

Using IBM SPSS Statistics version 25.0 (SPSS Inc., Armonk, NY, USA), survival percentages were analysed according to the NaOCl concentration and the altered parameters of the biofilm system in each experiment using two-way ANOVA. Differences between groups were assessed by an independent samples t-test or by ANOVA-testing followed by a Tukey post-hoc test analysis. The significance level was set at 0.05.

Electron microscopic imaging of the multispecies biofilms

Scanning electron microscopy (SEM)

Processing of samples for SEM analysis was performed following previously published protocols (Romero-Lastra *et al.* 2019). Dentine discs covered with biofilms grown for 11 days were fixed in 2.5% paraformaldehyde and 2% glutaraldehyde in 0.1M sodium cacodylate overnight at 4°C, then washed twice in 0.1M sodium cacodylate followed by 3 washes with water (immersion time 10 min). Then the biofilm was dehydrated through a series of graded ethanol solutions (50, 60, 70, 80, 90 and 100%; immersion time per series, 10 min). Then, samples were critical point dried (CPD 030; Bal-Tec, Balzers, Liechtenstein) and coated with a thin layer of platinum before being analysed with a scanning electron microscope (Zeiss Crossbeam 540; Zeiss GmbH, Oberkochen, Germany). Images were taken using a SE2 detector at 5 kV.

Transmission electron microscopy (TEM)

Multispecies biofilms (6 biological replicates) were grown for 11 days on aclar embedding film (EMS Cat.#50426-25; EMS, Hatfield, PA, USA). In addition, monospecies biofilms of the four

constituting species were grown on coverslips, as a positive control. Fixation was done in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M NaCacodylate buffer, pH 7.2 for 4 hours. Thereafter, cells were incubated in fresh fixative O/N at 4°C. After washing in buffer, they were post-fixed in 1% OsO₄ with 1.5% K₃Fe(CN)₆ in 0.1 M NaCacodylate buffer for 1 h. After washing, cells were subsequently dehydrated through a graded ethanol series, including a bulk staining with 1% uranyl acetate at the 50% ethanol step followed by embedding in Spurr's resin. To select the area of interest on the specimen block and in order to have an overview, semi-thin sections were first cut at 0.5 µm and stained with toluidin blue. Ultrathin sections of a gold interference colour were cut using an ultra-microtome (Leica EM UC6; Leica Biosystems, Vista, CA, USA), followed by a post-staining in a Leica EM AC20 for 40 min in uranyl acetate at 20 °C and for 10 min in lead stain at 20°C. Sections were collected on Formvar-coated copper slot grids. Grids were viewed with a JEM 1400plus transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV. Images were taken at random by an independent investigator at a magnification of 80x, 1000x, 5000x, 10000x and 50000x.

Results

Influence of the model system parameters on the NaOCl susceptibility of endodontic biofilms

Influence of atmosphere on the NaOCl susceptibility of *E. faecalis* biofilms

The mean number of CFU (expressed as log CFU/mL) did not differ significantly between the anaerobically incubated control group (7.77; SD 0.18) and the aerobically incubated control group (7.85; SD 0.13) ($P > 0.05$). The biofilm eradication after treatment with 0.025%, 0.1% and 0.5% NaOCl for the biofilms incubated in both atmospheres is shown in Figure 1A. There was no detectable survival in both groups after treatment with 2.5% NaOCl. The two-way ANOVA revealed that the NaOCl concentration, but not the oxygen presence significantly influenced the biofilm eradication ($P < 0.01$). Percentage biofilm survival decreased with increasing NaOCl concentration, with significantly different eradication results between all tested concentrations.

Influence of incubation time on the NaOCl susceptibility of *E. faecalis* biofilms

The mean number of CFU did not differ significantly between 24-hour grown untreated *E. faecalis* biofilms (7.77; SD 0.059) and 11-day-old untreated biofilms (7.51; SD 0.069) ($P > 0.05$). Biofilm eradication after treatment with 2.5%, 0.5%, 0.1% and 0.025% NaOCl for both incubation times is shown in Figure 1B. The two-way ANOVA revealed that both NaOCl concentration ($P = 0.001$) and incubation time ($P = 0.029$) significantly influenced the biofilm eradication. Also, a significant interaction between NaOCl concentration and incubation time was observed ($P = 0.001$), indicating that the effect of NaOCl concentration was different for both incubation times

(Figure 1B). For the one-day-old biofilms, a concentration-dependent biofilm eradication was seen, with higher NaOCl concentrations (0.5, 0.1, 2.5%) resulting in greater biofilm killing than 0.025% NaOCl ($P < 0.05$). For the 11-day-old biofilms, biofilm survival after treatment with 0.025, 0.1 and 0.5% was not significantly different ($P > 0.05$). It was observed that except for 0.025% NaOCl, 11-day old biofilms were less susceptible than 1-day old biofilms.

Influence of the biofilm composition on the NaOCl susceptibility of *E. faecalis*

The number of *E. faecalis* CFU was significantly lower in untreated monospecies biofilms (7.51; SD 0.069) than in untreated multispecies biofilms (8.05; SD 0.23) ($P < 0.05$). Biofilm eradication after treatment with 2.5%, 0.5%, 0.1% and 0.025% NaOCl for both biofilm compositions is shown in Figure 1C. The two-way ANOVA revealed that both NaOCl concentration ($P < 0.01$) and biofilm composition ($P < 0.01$) significantly influenced the biofilm eradication. A significant interaction between biofilm composition and NaOCl concentration ($P < 0.05$) was also observed. For all tested NaOCl concentrations, *E. faecalis* was less susceptible in the multispecies biofilm compared to the single species biofilm ($P < 0.05$).

Influence of the substrate on the NaOCl susceptibility of *E. faecalis* in a multispecies biofilm

The mean log CFU/mL in the control group for *E. faecalis* in a multispecies biofilm grown in MTP, on HA or on dentine was 8.05 (SD 0.23), 7.64 (SD 0.32), and 7.82 (SD 0.12), respectively. The difference in CFU counts between the MTP-grown biofilms and the HA-grown biofilms was significant ($P < 0.05$), other differences were not. Biofilm eradication after treatment with 2.5%, 0.5%, 0.1% and 0.025% NaOCl on different substrates is shown in Figure 1D. For NaOCl concentrations of 0.025% and 0.1%, the difference in susceptibility between MTP on the one hand and both the discs on the other hand was significant, with higher biofilm survival in the disc-grown biofilms. For the higher concentrations 0.5% and 2.5%, the same trend was observed but the difference was not significant. Both NaOCl concentration ($P < 0.01$) and biofilm substrate ($P < 0.01$) significantly influenced the biofilm eradication, and a significant interaction between both factors was revealed by two-way ANOVA. For all NaOCl concentrations, HA or dentine-grown biofilms displayed a lower NaOCl susceptibility compared to MTP-grown biofilms.

Multispecies biofilm model

Scanning electron microscopy

SEM images revealed a highly dense mass of bacterial cells that was homogeneous over the entire surface of the disc (Fig. 2B). Different bacterial morphotypes could be distinguished. The typical diplococ morphology of *E. faecalis* (Fig. 3.1, 3.2) and the typical fusiform morphology, long with tapered ends, of *F. nucleatum* (Fig.3.3,3.4) as seen on the TEM images of monospecies biofilms (Fig. 3 left panel), could be recognized as dominant organisms at the external surface of the biofilm (Fig. 2C).

Transmission electron microscopy

In the left panel of figure 3 the images of single species biofilms of *E. faecalis* (Fig. 3.1, 3.2), *F. nucleatum* (Fig. 3.3, Fig. 3.4), *P. gingivalis* (Fig. 3.5, Fig. 3.6) and *P. intermedia* (Fig. 3.7, Fig. 3.8) are shown. *E. faecalis* and *F. nucleatum* are clearly recognizable because of their typical cell morphology but this is not the case for *P. intermedia* and *P. gingivalis*, which have more similar morphological features. Both are short rods with similar size. In the right panel, the far right image shows a cross-section of an 11-day-old multispecies biofilm. The biofilm thickness ranged from 800 μm to 1200 μm (n=6). All the six imaged samples displayed a similar structure/architecture. Three regions could be discerned. The region closest to the substrate (Fig. 3G) contained a large number of dead cells and cell debris. Clusters of *F. nucleatum* were evident in this region, as well as dispersed *P. intermedia* or *P. gingivalis* cells. The central region (Fig. 3D) was characterized by a high cell density and fewer dead cells. All species, except *E. faecalis*, seemed to be present in similar proportions (Fig. 3E, F). In the outer layer, the most abundant species was *E. faecalis*, but *F. nucleatum* was also present, and the overall density of the biofilm was lower.

NaOCl susceptibility of the individual species in multispecies biofilm

An 11-day-old biofilm grown on a dentine discs was treated with different NaOCl concentrations (n=4*3). The log CFU of the individual species was quantified by plating the biofilm suspensions on selective media. In the control group (0% NaOCl) the mean log CFU for the individual species was 7.80 (SD 0.16) for *E. faecalis*, 7.38 (SD 1.1) for *P. intermedia*, 7.59 (SD 0.54) for *P. gingivalis*, and 7.16 (SD 0.20) for *F. nucleatum*. These numbers and their standard deviations demonstrate good reproducibility for this complex 11-day-old anaerobically grown multispecies community. After treatment with 2.5% NaOCl, the mean log CFU for the individual species was 5.04 (SD 1.07) for *E. faecalis*, 6.08 (SD 1.19) for *P. intermedia*, 6.04 (SD 1.56) for *P. gingivalis* and 6.24 (SD 0.32) for *F. nucleatum*. The high survival for all species (up to 5 log or more surviving cells) compared with the previous less complex models demonstrates the robustness of this model.

Discussion

A wide variety of biofilm model systems has been described in literature. Mostly, the focus of these laboratory studies has been on the antimicrobial efficacy of the tested treatment, whereas the model itself deserved little attention (Stojicic *et al.* 2013). This study focuses on the model system parameters *per se* and profoundly investigates a variety of parameters at the same time. The present work has clearly demonstrated that model parameters, such as the level of biofilm maturation, the composition of the biofilm and the substrate on which the biofilm is grown, do have a significant effect on the susceptibility of the biofilm bacteria to NaOCl. The consequences

of these findings are not to be underestimated. Since the present results indicate that the susceptibility of biofilms produced in relatively simple biofilm models is significantly higher compared to that of more complex and physiologically relevant model systems, the results of antimicrobial protocols in the former setup may represent an overestimation of the treatment effect, and hence lead to incorrect conclusions. A literature review highlighted the frequent use of a monospecies *E. faecalis* biofilm grown in single-rooted extracted teeth to evaluate the antimicrobial activity of endodontic irrigants or medicaments (Swimberghe *et al.* 2018). The present findings clearly demonstrate that *E. faecalis* is significantly less susceptible to NaOCl-treatment when grown in a multispecies community. This implicates that the antimicrobial action of irrigants/medicaments, obtained in models using monospecies *E. faecalis* biofilms, is overestimated, as most biofilms *in vivo* are polymicrobial. In this respect, the use of this simple and frequently used monospecies *E. faecalis* biofilm model to evaluate the antimicrobial activity of endodontic irrigants or medicaments, should no longer be encouraged.

In the first part of this study different biofilm set-ups were used. Each parameter was evaluated by comparing two biofilm set-ups, which differed from each other only in that specific parameter. Based on the findings, the best conditions were selected and subsequently incorporated in the following set-up. Thereafter, in the following experiment another parameter was evaluated. Basically, the first set-up was the most simplistic model system, and gradually various parameters were evaluated to end with the most relevant model in which the best conditions were used. In the second part of this study, this most relevant model system was further characterized.

The presence of oxygen did not affect the NaOCl susceptibility of *E. faecalis* biofilms, which is in agreement with Rashid *et al.* (2019). In this study, mixed-species biofilms grown anaerobically or aerobically did not differ significantly from each other after treatment with NaOCl. Because in subsequent experiments, *E. faecalis* had to be co-cultured with strictly anaerobic bacteria in biofilm set-up 4 (Table 2), and since anaerobic conditions are more prevalent in the necrotic root canals system, the anaerobic incubation was retained for all further experiments.

For all NaOCl concentrations, except 2.5%, a significant difference was seen between the 1-day and an 11-day-old *E. faecalis* biofilms. The 1-day-old biofilm displayed a concentration-dependent anti-biofilm action with complete eradication at 0.5% and above. However, this was not the case for the 11-day-old biofilm, where the concentration-dependent biofilm killing was not observed for 0.025%, 0.1% and 0.5% NaOCl, and treatment with 2.5% NaOCl still showed survival. Hence the biofilm age (incubation time) significantly affected the NaOCl susceptibility of *E. faecalis* biofilms, in the sense that older biofilms were less susceptible. These findings concur with previous reports on the relation between biofilm age and biofilm susceptibility. For example, Shen *et al.* (2011), using confocal microscopy and LIVE/DEAD staining, found that bacteria in mature (several

weeks) biofilms of (species) are more resistant to chlorhexidine than in young (2 days) biofilms . This finding was confirmed in a study of Stojicic *et al.* (2013), concluding that more mature multispecies biofilms grown from an oral sample are more resistant to a variety of disinfectants compared to young biofilms. Lim *et al.* (2009) reported that bacterial survival in 4-week-old *E. faecalis* biofilms was markedly greater than in the 4-day-old biofilms after treatment with light-activated disinfection and chemical disinfection. Chau *et al.* (2015) investigated the antibacterial activity of NaOCl on 22-, 46-, 70- and 96-h-old *E. faecalis* biofilms formed on hydroxyapatite discs. They observed that the antibacterial activity of NaOCl decreased with increasing biofilm age. These studies demonstrated that antimicrobial treatments are usually less effective against more mature *E. faecalis* biofilms. And therefore, in the subsequent biofilm set-ups it was opted to continue with an incubation time of 11 days.

Thirdly, the multispecies lifestyle decreased the NaOCl-susceptibility of *E. faecalis* biofilms. Several studies have shown that multispecies biofilms demonstrate increased tolerance to antimicrobial treatment compared to monoculture biofilms (Burmølle *et al.* 2006, Kara *et al.* 2006, Schwering *et al.* 2013). For example, Ozok *et al.* (2007) revealed that dual-species biofilms consisting of *Fusobacterium nucleatum* and *Peptostreptococcus micros* were less susceptible to NaOCl treatment than their monospecies counterparts. One of the mechanisms explaining the enhanced antimicrobial tolerance of polymicrobial communities is the difference in the composition of the extracellular polymeric substances (EPS) in multispecies biofilms, compared to that of monospecies biofilms (Bowen *et al.* 2018). In the present study, it was observed during the experiments that the wells with the multispecies biofilms contained a thick and dense red-brownish mass, whereas in the wells with the monospecies biofilms, a yellow-opaque and apparently less thick film was seen (data not shown). The thickness of the biofilms grown in the MTP was not determined and these visual observations were not further investigated, but could be an indication of a higher proportion of matrix in the biofilms grown on HA and dentine. This fits within the hypothesis proposed by Stewart & Costerton (2001), stating that the biofilm's increased tolerance to antimicrobial agents is in part due to the matrix, hindering or slowing the penetration of the AMA. A more abundant or differently composed biofilm matrix in this way could increase the reaction of NaOCl with matrix components and alter the NaOCl susceptibility of the biofilm. Also, in a multispecies community the spatial organization of microorganisms can lead to increased biomass and enhanced tolerance towards antibiotics compared to their component species individually (Kolenbrander *et al.* 2010, Lee *et al.* 2014, Liu *et al.* 2016). Given the polymicrobial nature of biofilms found in infected root canals, and the present findings demonstrating that multispecies biofilms are less susceptible to NaOCl treatment, the preference for a multispecies biofilm over a monospecies biofilm is obvious.

The substrate on which the biofilm was grown was the final parameter to be evaluated. Although cell numbers in the MTP biofilm (*E.f.*: 8.04 log units, total count: 8.30 log units) were slightly higher than in the HA (*E.f.*: 7.64 log units, total count: 7.82 log units) and dentine biofilm (*E.f.*: 7.82 log units, total count: 8.16 log units), it was observed that the NaOCl susceptibility of *E. faecalis* grown in a multispecies biofilm in a MTP (2.5% NaOCl: 2.8 log units) was higher compared to the same biofilm cultured on HA (5.01 log units) or dentine (5.56 log units). In addition, the disc-grown biofilms were treated with a 3 times greater NaOCl volume than the MTP-grown biofilms. This was necessary in order to completely submerge the disc with biofilm in the treatment solution. Consequently more active chlorine was available to attack the disc-grown biofilms. So although the difference in NaOCl susceptibility was not significant for all concentrations, the fact that a greater volume of disinfectant was used on the disc-grown biofilms further supports this trend of higher survival of the biofilms grown on hydroxyapatite or dentine. For all concentrations the percentage survival was similar for HA and dentine. These findings are in line with the study of Lukic *et al.* (2020) in which an endodontic multispecies biofilm was grown on pellicle-coated HA and dentine discs. Quantitative analysis determined by quantitative real-time PCR showed similar cell counts in both biofilms. In another study (Guerreiro-Tanomaru *et al.* 2013), using confocal microscopy, *E. faecalis* biofilm formation on different substrates, including dentine and HA, was analysed. They concluded that HA was the substrate with the best conditions for biofilm development. However, in the present experiment no significant differences in cell counts between HA- and dentine-grown biofilms were observed. It has been established that the formation and structural organization of a biofilm are influenced by the chemical nature of the substrate (Stepanovic *et al.* 2004). The substratum will not only influence the initial adhesion of colonizing cells, but it will also influence the production of signalling molecules that control cell physiology and virulence (Chávez de Paz *et al.* 2010). To mimic the *in vivo* situation more closely and to enhance the adherence of the bacteria, substrates are often coated with various substances such as collagen, saliva, bovine serum albumin or mucin (Shen *et al.* 2009, Lundstrom *et al.* 2010, Halford *et al.* 2012, Schaudinn *et al.* 2013, Layton *et al.* 2015). Chávez de Paz *et al.* (2010) revealed that a different biofilm structure was formed on a collagen-conditioned surface compared to a clean polystyrene surface. It is clear that the microtiter plate material used in this set of experiments does not represent the root canal surface. So, it is likely that a different biofilm structure will be formed on HA and dentine than on the polystyrene surface of the MTP. In preliminary experiments the influence of surface coating of HA (data provided in supportive information S2) was examined. HA discs were preconditioned with collagen, saliva or BHI. Since no difference in biofilm cell counts between the 3 coatings could be observed, the BHI was further used. Also, the dentine discs were pretreated with EDTA to enhance the adhesion of the

bacteria to the surface. It was shown that demineralization of dentine by EDTA exposes the collagen and creates an ideal substrate for adherence by *E. faecalis* (Kishen et al. 2008). Collagen in dentine may also serve as an adhesion substrate for other bacteria (Switalski et al. 1993).

In an effort to mimic the *in vivo* endodontic biofilm, physiologically relevant model parameters were incorporated in a complex biofilm model system. The developed model was comprised of an 11-day-old multispecies biofilm of *E. faecalis*, *F. nucleatum*, *P. gingivalis* and *P. intermedia* grown on HA or dentine discs under anaerobic conditions. A requirement of such an engineered biofilm model system is that the individual species are still proportionally represented after a sufficiently long incubation period. It is a challenge to find media and conditions that simultaneously support biofilm formation of multiple species, while avoiding that one species outcompetes or inhibits the others (Gabriliska & Rumbaugh 2015). Although in the infected root canal, the individual species are not present in equal numbers, in a biofilm model system this is to some degree desirable because it enhances the ability to quantify the effects of a treatment on the individual species. A pilot study had shown that when combining the four species from the beginning, *E. faecalis* dominated the other species, resulting in a biofilm with very low numbers of the three anaerobic species. These findings of *E. faecalis*-associated suppression of growth of other species within a multispecies biofilm are in line with the results of previous studies (Chávez de Paz et al. 2015, Lukic et al. 2020). Therefore, in further exploratory experiments, *E. faecalis* was added in different concentrations and at different time points. Finally, the inoculation, as described in the materials & methods section, adding *E. faecalis* at day 8 of the incubation, resulted in a multispecies model with all members of the community being present.

The complex, but physiologically relevant, biofilm model was then further characterized using electron microscopy techniques and used to evaluate the influence of NaOCl treatment on each species of the mixed community. In contrast to quantification methods resulting in total counts (i.e. the sum of all constituting species), this method enables monitoring of the effect of a treatment on each individual strain in the multispecies biofilm.

Biofilm formation was confirmed by SEM. These SEM images show a dense mass of a mixed bacterial community covering the discs. In the top layer, *E. faecalis* and *F. nucleatum* could be clearly identified. However, SEM only allows an appreciation of the external biofilm surface. Therefore TEM was included to get further insight into the biofilm architecture and the spatial distribution of the species in the biofilm. Three different zones could be recognized, demonstrating that the biofilm composition was different at varying depths. The proportion of dead cells in the innermost layer was striking. *F. nucleatum* was clearly distinguished by its morphology and was found in all layers of the biofilm. This anaerobic bacterium is known as a

bridge-building organism that coaggregates with initial, early and late colonizers and therefore plays an essential role in multispecies biofilm formation (Kuboniwa *et al.* 2009, Kolenbrander *et al.* 2010). *E. faecalis* was exclusively present in the top layer. This is most likely a consequence of the two-phase inoculation. Based on the TEM images it was almost impossible to make a distinction between *P. gingivalis* and *P. intermedia*. Mainly, these bacteria could be found in the centre of the biofilm and to a lesser extent in the area close to the substrate. Again these findings comply with the hypotheses proposed by Stewart *et al.* (2001). The multicellular nature of the biofilm displays an altered microenvironment. Oxygen consumption in the surface layers of the biofilm will lead to anaerobic niches in the deep layers, where strict anaerobic bacteria, such as *P. gingivalis* and *P. intermedia*, can be found. Although *F. nucleatum* is also an anaerobe, its capacity to adapt to an oxygenated environment is extremely high (Diaz *et al.* 2002) and therefore it is also present in the upper layers of the multispecies biofilm.

In the present model, biofilms are formed on a disc, a setup that lacks the confinement and configuration of a root canal, thus representing a potential limitation. On the other hand, the high degree of standardization of this model excludes disturbing anatomical variability and allows to focus on the interaction between the disinfectant and the biofilm. Future efforts will focus on translating the present model into a root canal geometry. When working with extracted teeth, additional complexity is added to the experimental model, and this comes with additional challenges and difficulties. For example, robust determination of remaining/surviving biofilm in extracted teeth is a lot more complicated compared to working with discs or wells. Whether biofilms should be grown in single-rooted teeth or in teeth with more complex anatomy, is dependent on the research question. The ultimate goal should be to evaluate irrigants or medicaments in a multispecies biofilm grown in extracted teeth with a complex canal anatomy. This model is the closest possible to the clinical reality, but also the most complex, technically demanding and time-consuming. Experimental setups with less complexity could however still be meaningful, as they might provide insights into working mechanism or partial aspects. In this respect, the monospecies *E. faecalis* model could still be useful as a tool to screen certain AMA before going to more complex models.

Another potential drawback might be the rather short contact time between the disinfectant and the biofilm in the present study. However, it was seen in preliminary experiments that longer contact times led to high biofilm eradication (especially in the less complex set-ups), but also more variable results. It can also be argued that the disc model is an optimized situation in the sense that there is a great contact area between the biofilm and the disinfectant and optimal exposure. Contact times between NaOCl and the intracanal microbiota in the clinical situation are longer, but within the confined space of the root canal, the volume of NaOCl is small and the

contact with the biofilm is limited (Petridis *et al.* 2019). This warrants the use of a shorter contact time. Although only NaOCl was tested as disinfectant in the present study, this biofilm model has potential for assessment of a variety of agents used for root canal disinfection.

Conclusion

The parameters biofilm age, biofilm composition and substrate had a significant influence on the NaOCl susceptibility of the biofilm. Longer incubation time, multispecies composition and hydroxyapatite or dentine substrates resulted in biofilms that were less susceptible to NaOCl treatment. These findings emphasize the importance of selecting relevant parameters when designing and choosing a biofilm model system for downstream evaluation of antimicrobial agent efficacy.

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Conflict of Interest statement

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

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Table 1 Overview of strains used in this study, isolation sites, oxygen preference and selective medium

Species name	Strain nr	Origin	Oxygen preference	Medium
<i>Enterococcus faecalis</i>	LMG 8222 (ATCC 29212)	Human urine	Facultative anaerobic	Brain Heart Infusion agar
<i>Fusobacterium nucleatum</i>	DSM 19507 (ATCC 49256)	Human periodontal pocket	Strict anaerobic	Crystal Violet-Erythromycin agar
<i>Porphyromonas gingivalis</i>	DSM 28984	Human oral cavity	Strict anaerobic	<i>Porphyromonas Gingivalis</i> agar supplemented with mupirocine
<i>Prevotella intermedia</i>	ATCC 49046	Human periodontal pocket	Strict anaerobic	Schaedler Kanamycin/Vancomycin agar

Table 2 Overview of the different biofilm set-ups. The parameter that is altered compared to the previous set-up is shown in bold.

	Atmosphere	Incubation time	Composition	Substrate
Biofilm 1	aerobic	1 day	monospecies	MTP
Biofilm 2	anaerobic	1 day	monospecies	MTP
Biofilm 3	anaerobic	11 days	monospecies	MTP
Biofilm 4	anaerobic	11 days	multispecies	MTP
Biofilm 5	anaerobic	11 days	multispecies	HA
Biofilm 6	anaerobic	11 days	multispecies	dentine

Figure legends

Figure 1 Biofilm survival after treatment with different NaOCl concentrations under various conditions, expressed as percentage (logarithmically scaled) relative to untreated controls. Bars show mean values and vertical error bars indicate standard deviations, $n \geq 3 \times 3$. Horizontal brackets indicate significant differences between groups ($P < 0.05$). A) 24-h-old *E. faecalis* biofilms incubated under anaerobic (ANAER) or aerobic (AER) conditions. B) 1-day or 11-days-old *E. faecalis* biofilms incubated under anaerobic conditions. C) *E. faecalis* in a monospecies or multispecies biofilm incubated for 11 days under anaerobic conditions. D) *E. faecalis* in multispecies biofilm grown in MTP, on HA or dentine and incubated for 11 days under anaerobic conditions.

Figure 2 A dentine disc covered with an 11 day-old multispecies biofilm in a well with culture medium (A). Scanning electron microscopy image of the same biofilm at 2000x (B) and 10 000x (C) magnification.

Figure 3 Left: TEM images of 11-day-old monospecies biofilms of *E. faecalis* (1,2), *F. nucleatum* (3,4), *P. gingivalis* (5,6) and *P. intermedia* (7,8). Right: The far right image shows a cross-section of the entire multispecies biofilm grown for 11 days. These micrographs show the distribution of the different species within the biofilm.

Figure 4 The log CFU of the individual species in a 11-day-old multispecies biofilm grown on dentine discs. Biofilms were treated with 0%, 0.025%, 0.1%, 0.5% and 2.5% NaOCl. Graphs show means and standard deviations. EF = *E. faecalis*, PI = *P. intermedia*, PG = *P. gingivalis* and FN = *F. nucleatum*. $n=4 \times 3$

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iej_13544_f3.tif

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