Insights into passive ultrasonic irrigation

Jiang, L.M.

9

Resazurin metabolism assay for root canal disinfectant evaluation on dual-species biofilms

This chapter has been published as: Jiang LM, Hoogenkamp MA, van der Sluis LW, Wesselink PR, Crielaard W, Deng D. Resazurin metabolism assay for root canal disinfectant evaluation on dual-species biofilms. J Endod. 2011 Jan;37(1):31-5

Abstract

Introduction: Endodontic infections are caused by polymicrobial biofilms. Novel root canal disinfectants therefore should be evaluated not only on single-species biofilms but also on dual- or mixed-species biofilms. A simple, high throughput assay is urgently needed for this. In this study the application of the resazurin metabolism assay was investigated for evaluation of a root canal disinfectant on dual-species biofilms. **Methods:** *Enterococcus faecalis* with or without *Streptococcus mutans* in biofilms were formed in an active attac0hment biofilm model for 24h. Subsequently, the biofilms were treated with various concentrations of NaOCl for 1 min. After resazurin metabolism by both organisms was confirmed, treatment efficacies using 0.0016% resazurin were evaluated. **Results:** During NaOCl treatments, resazurin metabolism displays a clear dose response, not only in single-species *E. faecalis* (or *S. mutans)* biofilms, but also in dual-species biofilms. Notably the assay revealed that the resistance of dual-species biofilms to NaOCl was 30 fold higher than in single-species *E. faecalis* biofilms. Viability counts on a selected NaOCl treatment (0.004%) confirmed this result and showed the increased resistance of *E. faecalis* in dual-species biofilms. **Conclusions:** Clearly the high throughput and low cost resazurin metabolism assay has a great potential for testing novel root canal antimicrobial agents in mixed-species biofilms.

Introduction

Biofilms, by definition, are matrix-enclosed microbial communities in which cells adhere to each other and/or to surfaces or interfaces (1, 2). It represents the prevalent mode of microbial life in nature, industrial processes and infections (3). Also the relation between biofilms and endodontic infections has recently been revised by Svensäter et al. (4).

In biofilm research viability testing, using colony forming unit (CFU) counts, is frequently applied. This method is laborious, time consuming and not suitable for high-throughput screening. Moreover biofilms have to be efficiently removed from surface and dispersed before plating and the efficacy of this process is often in question. Simpler methods, for example fluorescence live/dead cell staining and metabolic activity indicators, have previously been suggested (5) since they could be high-throughput and avoid the biofilm detachment/dispersal steps.

Resazurin is one of the common metabolic activity indicators (6). It is a nontoxic, water-soluble dye (blue and non-fluorescent) which can be reduced to water-soluble resorufin (pink and highly fluorescent) by metabolically active bacteria (7, 8). It has been used to assess planktonic bacterial viability, adaptation to stress and bacterial contamination (9-11). Recently it was also explored for its potential of biofilm quantification in microtiter plates (5) and from six assays tested, it was suggested as one of the best alternatives for CFU counts. It was also successfully used to evaluate disinfection efficacy on single-species biofilms of several clinically relevant (non-oral) pathogenic bacteria (6, 12). It has, however, never been used in dual- or mixed-species biofilms.

In the recent years, many researchers started to validate new endodontic disinfectants in single-species biofilm models (13-15). It is however well established that endodontic infections are typically polymicrobial (16). Microbe-microbe interactions may increase total biofilm formation (17) and change the characteristics of a biofilm which may alter the resistances pattern to antimicrobials (18). Our previous study has shown that *Enterococcus faecalis* E2 forms more biofilms in the presence of *Streptococcus mutans* UA159 (17). It is of our interest to study whether this will also influence the antimicrobial resistance of *E. faecalis* E2.

The aim of this study is twofold, firstly to explore the application of the resazurin metabolism assay for evaluation of the root canal disinfectant NaOCl on dual-species biofilms and secondly to test if an increase in biofilm formation of an *E. faecalis* clinical strain by microbe-microbe interactions, shown in an earlier study, will influence its resistance to NaOCl.

Therefore *S. mutans* and *E. faecalis* dual-species biofilms were grown in a similar way as in our previous study (17). We chose *S. mutans* because *Streptococcus* species were reported to be present in 47% of root-canal treated teeth when 16s rRNA probes are used (19), *S. mutans* has been detected in root canal infections (20, 21) and it is a strong biofilm former. Since *S. mutans* biofilm formation is most potent in the presence of sucrose, this carbohydrate were used as the energy source in the biofilm experiments.

Materials and Methods

Bacterial strains and growth conditions

Clinical *E. faecalis* strain E2 (kindly given by Dr. C.M. Sedgley) (22) and *S. mutans* strain UA159 were used in this study. Both strains were routinely grown anaerobically on Brain Heart Infusion (BHI) agar at 37 °C. Biofilms were grown in modified semi-defined biofilm medium (BM), as described previously (17). The pH of this medium was 7.0.

Biofilm Assays

Biofilms were grown in an active attachment Calgary biofilm model. This model consists of a standard 96-well microtiter plate and a lid with an identical number of polystyrene pegs that fit into the wells (NuncTM, Roskilde, Denmark) (23, 24). It was chosen to avoid potential bacterial sedimentation in the flat-bottom 96-well microtiter plate but to remain the 96-well high-throughput advantage for disinfection treatment assays.

E. faecalis biofilms were grown anaerobically on pegs as single-species or together with preformed 24-hour *S. mutans* biofilms. In detail: one *S. mutans* UA159 colony was inoculated into BM medium with addition of 0.36% glucose and incubated overnight. The overnight culture was diluted to a final OD_{600} of 0.016 in fresh BM medium with addition of 0.2% sucrose and 200 µL of the cell suspension was dispensed into a sterile 96-well plate. An equal number of wells were also filled with 200 µL of the fresh BM medium. The plate was then covered with a sterile lid containing 96 pegs. After 24-hour incubation, the pegs with or without *S. mutans* biofilms were rinsed with phosphate buffered

saline (PBS) solution to remove non-adherent bacterial cells and were subsequently used as substrata for *E. faecalis* biofilm formation. One *E. faecalis* E2 colony was inoculated into BM with 0.36% glucose and incubated overnight. This culture was then diluted to an OD_{600} of 0.06 in fresh BM medium with 0.2% sucrose and 200 µL of the cell suspension was dispensed into a new sterile 96-well plate. The pegs with or without 24-hour *S. mutans* biofilms were inserted in the cell suspension. After 8-hour incubation, the pegs were rinsed with PBS and placed in fresh BM (with 0.2% sucrose). After another16-hour incubation, NaOCl treatments were applied on the single-species *E. faecalis* and dual-species biofilms in parallel. A 24-hour *S. mutans* biofilm also grew further without the presence of *E. faecalis* E2 and was then subjected to similar measurements and treatments.

For one experiment, one and a half 96-well plates were needed to test all single/dual-species biofilms. Forty-four single/dual-species biofilms were formed. The rest of the wells were used for no-bacterial controls. Maximum 10 NaOCl concentrations and treatment control were tested on 4 biofilm replicates. Each experiment was repeated three times.

NaOCl treatment on single- and dual-species biofilms

Pegs with biofilms were first rinsed with PBS and then inserted into new 96-well plates filled with 10 serial two-fold diluted NaOCl solutions (200 µL/well) or Milli-Q water (as a treatment control) for 1min. The NaOCl solutions were diluted from a stock solution with 8% active chlorine with Milli-Q water. Active chlorine concentrations in the NaOCl solutions were verified by iodine titration (25). Treatments were stopped by inserting the biofilm pegs in neutralizer (buffered peptone water with 0.1% sodium thiosulfate) for 5 min (18). The treatment control group was also subjected to the neutralizer incubation.

Evaluation on NaOCl treatment efficacy

Resazurin metabolism assay. To establish if resazurin is metabolized by and hence can be used as a metabolic activity indicator for *S. mutans* or *E. faecalis*, serial dilutions of planktonic *S. mutans* or *E. faecalis* cultures were assayed with resazurin. To this end, two milliliter overnight cultures of *E. faecalis* E2 or *S. mutans* UA159 in BM supplemented with 0.36% glucose were centrifuged (2 min, 14000 rpm) and the resulting pellet was washed and resuspended in 2 ml of BM-YE (BM without yeast extract). The yeast extract was removed from BM to prevent bacterial growth. Individual cultures were then two-fold diluted in BM-YE and dispensed at 180 µL/well into a 96-well plate in quadruplicate. A resazurin stock solution was added to achieve a final concentration of 0.0016%. The fluorescence intensity (FI) of each well was recorded at room temperature in a fluorimeter (Spectramax M2, Molecular Device, Sunnyvale, California), using the fluorescence setting with 485 nm excitation- and 580 nm emission wavelength. This experiment was performed in duplicate.

When evaluating biofilms, the resazurin stock solution was diluted in BM-YE to 0.0016% and dispensed at 200 µL/well into a 96-well plate. The pegs with biofilms after NaOCl treatments were inserted into the resazurin and the FIs of the resazurin solutions were measured at 2h in the fluorimeter. For all resazurin measurements, a background control group where 0.0016% resazurin was mixed with sterile BM-YE was included to record background FI changes, which were subsequently subtracted from the FIs. Treatment efficacy was calculated as the percentage reduction in FI values after treatment relative to the values in the control group.

Furthermore, a pilot study had shown that the neutralizer has no influence on resazurin FI values (data not shown).

Sonication-colony forming unit counts assay. Where indicated the sonication-CFU counts assay was also applied to test the treatment efficacy on the biofilms. Each individual peg was carefully cut with a sterile scalpel without disturbing the biofilms and placed in 1ml Cysteine Peptone Water (CPW)(24). Biofilms were dispersed by sonication on ice for 60 times 1 second at an amplitude of 40 W (Vibra cell^{TM}, Sonics & Materials Inc., USA). Serially diluted samples were plated onto BHI agar plates. The plates were incubated anaerobically at 37 ℃ for 2 days and colonies were counted.

Statistical analysis

Data were analyzed with the Statistical Package for Social Science (Version 17.0, SPSS, Chicago, IL, USA). One-way ANOVA was used to evaluate the effects of treatments and biofilm types (single-species or dual-species) on CFU counts. CFU counts were log transformed before the statistical tests. *P* value <.05 was considered as significant.

Results

Resazurin metabolism by S. mutans and E. faecalis planktonic cells

Figure 1 shows the Fluorescence intensity (FI) values after resazurin metabolism (after 2 h) in comparison with CFUs present in the assay (Fig. 1*A E. faecalis* and 1*B S. mutans*). A linear relationship was found for both species with good correlation efficiencies of 0.9 ($p=0.002$; *E. faecalis*) and 0.97 ($p<0.01$; *S. mutans*) at the shown range of bacterial cell numbers.

Evaluation of NaOCl treatment efficacy by resazurin metabolism and sonication-colony forming unit counts

In a pilot study, we determined the growth conditions for biofilm formation. Under the conditions mentioned in the Materials and Methods (including the inoculum size and medium refreshment frequency), biofilm formation was reproducible and on average $5x10^7$ CFU/ml viable cells were found on each peg.

In figure 2*A* and 2*B*, the percentage of FI reduction relative to the non-treatment group is plotted. Clear dose responses were seen in all types of biofilms. Single-species *E. faecalis* biofilms appeared to be about 30 times more sensitive to the NaOCl treatment than single-species *S. mutans* or dual-species biofilms (Figure 2*B*). No clear differences in sensitivity to NaOCl treatment was seen between the last two types of biofilms (Figure 2*A*). This experiment shows that the NaOCl concentration needed for more than 90% metabolism inhibition was 0.0039% for single-species *E. faecalis* biofilms and 0.125% for single-species *S. mutans* and dual-species biofilms.

More *E. faecalis* cells were present in the dual-species biofilms than in the single-species *E. faecalis* biofilms (as was also shown previously (17)). Figure 3 shows that 0.004% NaOCl significantly reduced viable cell numbers in all types of biofilms. Clearly this reduction is significantly greater in *E. faecalis* single-species biofilms than in *E. faecalis – S. mutans* dual-species biofilms ($p<0.05$).

Discussion

Our data revealed the clear application potential of the resazurin metabolism assay for evaluating efficacy of novel root canal disinfectants on both single- and dual- or mixed-species biofilms. First of all, at the indicated range of bacterial cell numbers, FI value changes resulting from resazurin to resorufin metabolism were linearly correlated with CFU counts for both *S. mutans* UA159 and *E. faecalis* E2. A similar linear correlation was also found for other clinical *E. faecalis* strains (data not shown). Secondly, during NaOCl treatments, which are commonly used for root canal disinfection, the resazurin metabolism assay showed clear dose responses not only for single-species *S. mutans* or *E. faecalis* biofilms but, more importantly, also for dual-species *S. mutans* and *E. faecalis* biofilms. Thirdly, the resazurin assays showed that the resistance to NaOCl of the dual-species biofilms was 30 fold higher than *E. faecalis* single-species biofilms. The viability counts on the chosen NaOCl treatment (0.004%) confirmed this result. Furthermore, our viability counts indicated that the presence of pre-formed *S. mutans* UA159 biofilms resulted in not only increased biofilm formation of *E. faecalis* E2 strain, as shown previously, but also the increased NaOCl resistance of this strain. This increased resistance towards NaOCl of *E. faecalis* in dual species biofilm is similar to what was found in other studies (18, 26)

The resazurin metabolism assay is a high throughput, simple and low-cost assay. As shown in our study the resazurin assay can be applied in a 96-peg biofilm model. Different antimicrobial agents in various concentrations can be tested in one 96-well plate and results can be achieved in 2 hours. We choose to use fluorescence intensity changes instead of absorbance changes as the read-out parameter since background signals are lower during FI changes. Nevertheless, in a setting where only a UV/VIS spectrophotometer is available, application of the resazurin assay is still possible (27). The resazurin assay does not require removal of the biofilms from the substratum, which avoids common problems encountered during traditional viability tests. In these tests, complete removal of the biofilms is often questionable and cells might not be fully dispersed or could be damaged during sonication.

None of the disinfection assays currently available for oral microbiology field is perfect. This also applies to the resazurin metabolism assay. Several limitations became apparent during our study: (i) There is a narrow detection/application range of 10^5-10^7 CFU/ml in the assay-well. (ii) Although it has been shown that a broad spectrum of bacteria can metabolize resazurin, applicability has to be tested for every new bacterial species and again the detection/application range for this species has to be evaluated. (iii) In dual-species or mixed-species biofilms the resazurin metabolism assay is not able to distinguish viability changes of the individual species.

Therefore, when evaluating novel disinfection regimen, the resazurin metabolism assay is typically ideal as an *initial* screening method to determine various treatment conditions (concentrations, treatment durations, etc). If necessary, CFU assays can subsequently be used to answer detailed questions, like how is which bacterial species affected in mixed-species biofilms.

In the current study we found that the resistance to NaOCl of single-species *S. mutans* biofilms was comparable to that of the dual-species biofilms. A possible explanation for this could be that in our experimental setting, the dual-species biofilms were grown for 24 hours while *S. mutans* biofilms grown for 48 hours. The maturation status of the cells in biofilms might be related to their antimicrobial resistance (28). It is also likely that the antimicrobial resistance is related to the amount of biofilm biomass, rather than on bacterial interactions in the biofilms. The fact that the single-species *E. faecalis* biofilms, which contain less biomass than the single-species *S. mutans* biofilms and the dual-species biofilms, also shows the highest sensitivity can also be explained by biomass related antimicrobial resistance (29). Further studies are needed to obtain a better understanding of the mechanism(s) underlying the resistance difference between single- and dual-species biofilms.
Since evidence for antimicrobial resistance of mixed-species biofilm cells is accumulating, it is apparent that traditional and novel root canal disinfection and treatment strategies will have to be evaluated on multi-species biofilm models. A high throughput strategy is urgently needed for this type of studies and our data indicate that resazurin metabolism assay has great potential in serving this purpose in mixed-species infections.

Figure 1. Relationship between resazurin metabolism activity and planktonic cell counts in *S. mutans* and *E. faecalis*. ***A*** Fluorescence intensity values of *S. mutans* UA159 at 2h were plotted against the viable counts. ***B*** Fluorescence intensity values of *E. faecalis* E2 at 2h were plotted against the viable counts. AU refers to arbitrary units.

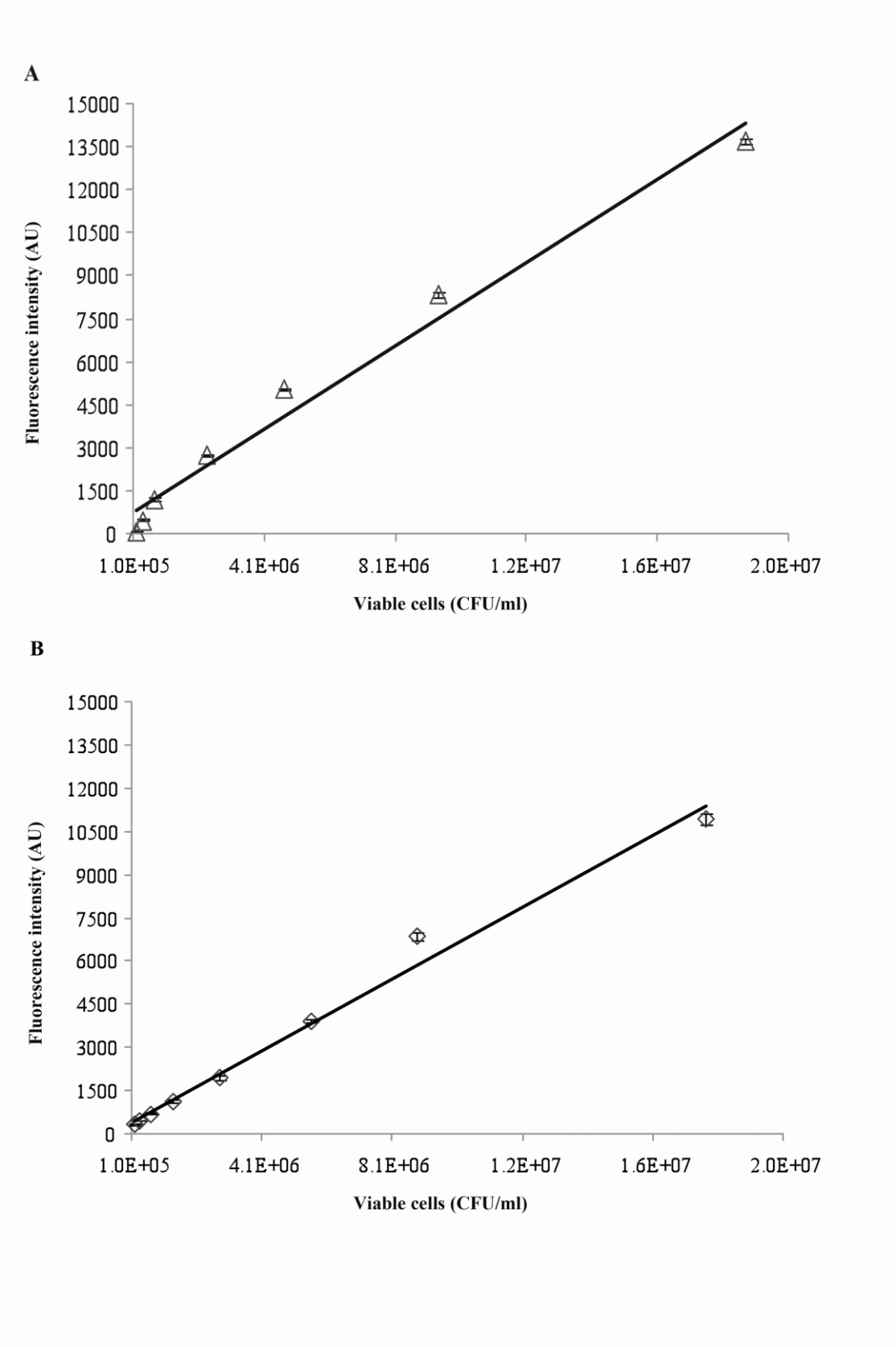
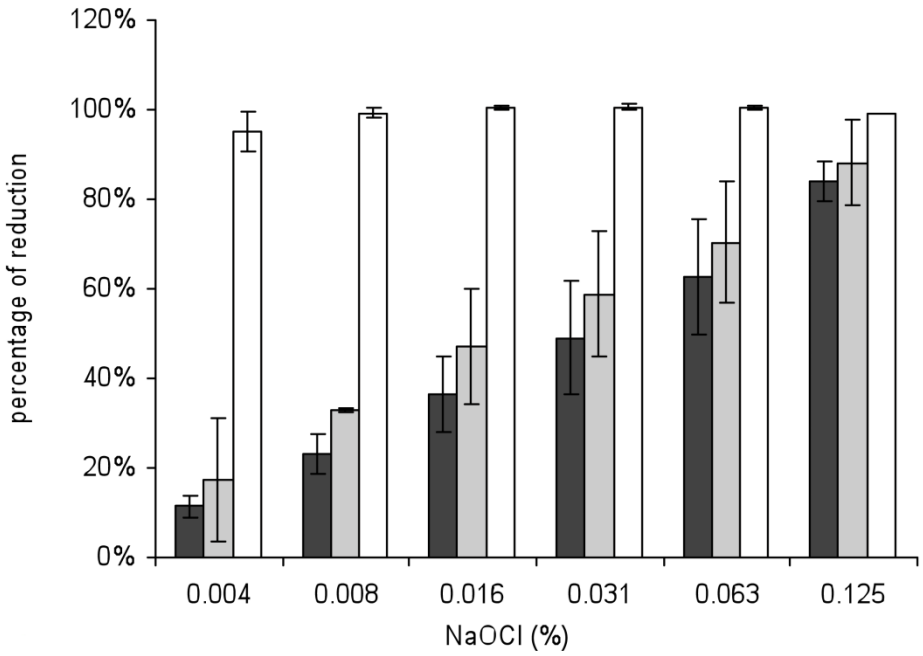


Figure 2. Efficacy of NaOCl treatments on single-species *S. mutans* or single-species *E. faecalis* biofilms and dual-species *S. mutans*-*E. faecalis* biofilms. White bars represent single-species *E. faecalis* biofilm, grey bars represent single-species *S. mutans* biofilm and dark grey bars represent dual-species *S. mutans*-*E. faecalis* biofilms. The efficacy of the treatment was evaluated by resazurin assay and presented as the percentage of FI reduction relative to the non-treatment group. ***A*** High NaOCl concentration range in single- and dual-species biofilms. ***B*** Low NaOCl concentration range for single-species *E. faecalis* biofilms.

A



B

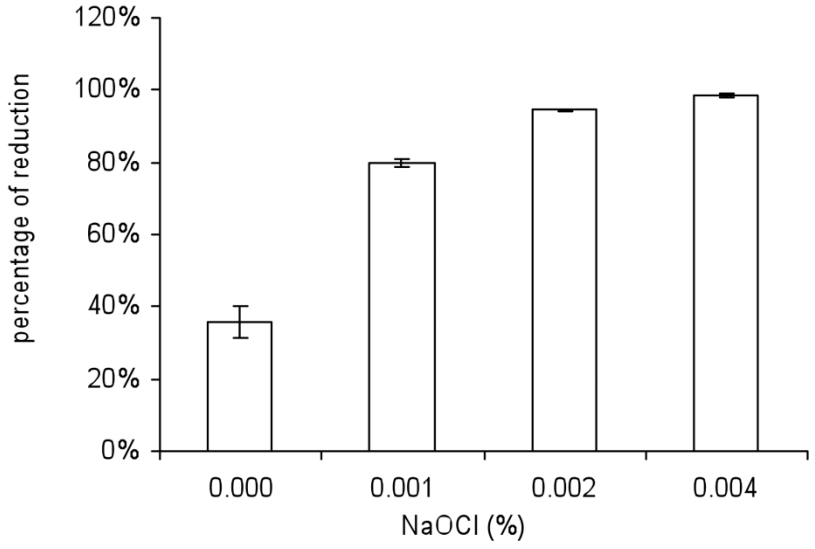
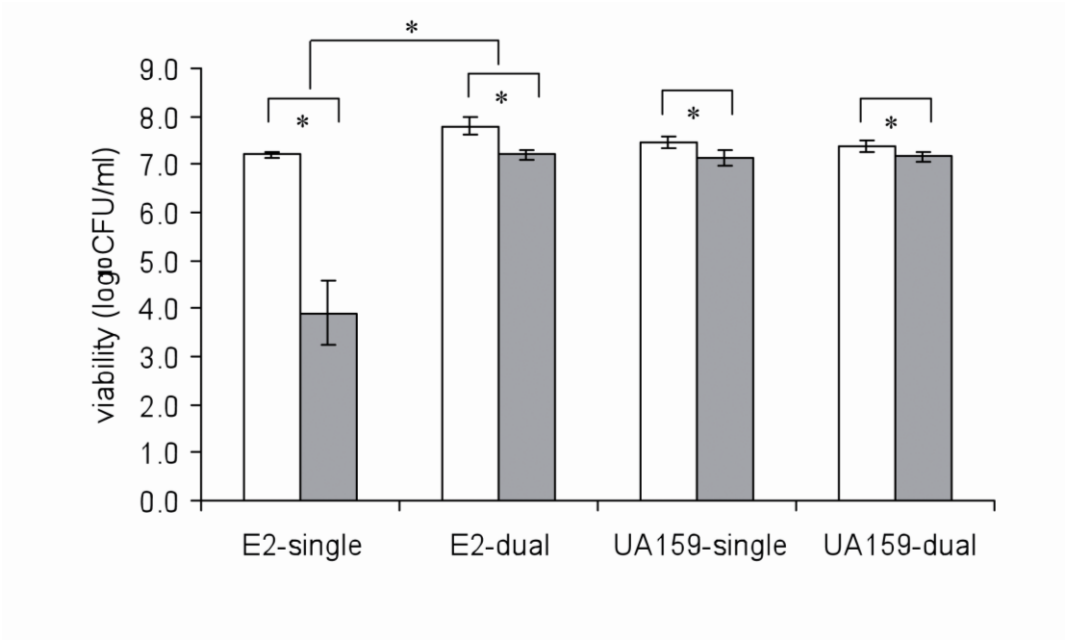


Figure 3. Viability of *S. mutans* UA159 and *E. faecalis* E2 in single-species and dual-species biofilms after a 0.004% NaOCl treatment. The viability is presented as $\log_{10}$CFU/ml. Significant reductions in viability (white bars represent before treatment, grey bars represent after treatment) are seen for both species, irrespective of the number of species in the biofilms. The reduction of *E. faecalis* E2 strain is significantly higher in single-species biofilm than in the dual-species biofilms. * indicates significance ($p<0.05$).



References

1. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002;15:167-93.
2. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annu Rev Microbiol 1995;49:711-45.
3. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2004;2:95-108.
4. Svensäter G, Bergenholtz G. Biofilms in endodontic infections. Endodontic Topics 2004;9:27–36.
5. Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods 2008;72:157-65.
6. Peeters E, Nelis HJ, Coenye T. Evaluation of the efficacy of disinfection procedures against Burkholderia cenocepacia biofilms. J Hosp Infect 2008;70:361-8.
7. Sreenivasan PK, Tambs G, Gittins E, Nabi N, Gaffar A. A rapid procedure to ascertain the antimicrobial efficacy of oral care formulations. Oral Microbiol Immunol. 2003;18:371-8.
8. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem. 2000;267:5421-6.
9. Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods 2007;42:321-4.
10. Deng DM, ten Cate JM, Crielaard W. The adaptive response of Streptococcus mutans towards oral care products: involvement of the ClpP serine protease. Eur J Oral Sci 2007;115:363-70.
11. Shiloh MU, Ruan J, Nathan C. Evaluation of bacterial survival and phagocyte function with a fluorescence-based microplate assay. Infect Immun 1997;65:3193-8.
12. Mariscal A, Lopez-Gigosos RM, Carnero-Varo M, Fernandez-Crehuet J. Fluorescent assay based on resazurin for detection of activity of disinfectants against bacterial biofilm. Appl Microbiol Biotechnol 2009;82:773-83.
13. Chavez de Paz LE, Bergenholtz G, Dahlen G, Svensater G. Response to alkaline stress by root canal bacteria in biofilms. Int Endod J 2007;40:344-55.
14. Chai WL, Hamimah H, Cheng SC, Sallam AA, Abdullah M. Susceptibility of Enterococcus faecalis biofilm to antibiotics and calcium hydroxide. J Oral Sci 2007;49:161-6.
15. Giardino L, Ambu E, Savoldi E, Rimondini R, Cassanelli C, Debbia EA. Comparative evaluation of antimicrobial efficacy of sodium hypochlorite, MTAD, and Tetraclean against Enterococcus faecalis biofilm. J Endod 2007;33:852-5.
16. Chavez de Paz LE. Redefining the persistent infection in root canals: possible role of biofilm communities. J Endod 2007;33:652-62.
17. Deng DM, Hoogenkamp MA, Exterkate RA, Jiang LM, van der Sluis LW, Ten Cate JM, et al. Influence of Streptococcus mutans on Enterococcus faecalis biofilm formation. J Endod 2009;35:1249-52.
18. Ozok AR, Wu MK, Luppens SB, Wesselink PR. Comparison of growth and susceptibility to sodium hypochlorite of mono- and dual-species biofilms of Fusobacterium nucleatum and Peptostreptococcus (micromonas) micros. J Endod 2007;33:819-22.
19. Rocas IN, Hulsmann M, Siqueira JF, Jr. Microorganisms in root canal-treated teeth from a German population. J Endod 2008;34:926-31.
20. Munson MA, Pitt-Ford T, Chong B, Weightman A, Wade WG. Molecular and cultural analysis of the microflora associated with endodontic infections. J Dent Res 2002;81:761-6.
21. da Silva LA, Nelson-Filho P, Faria G, de Souza-Gugelmin MC, Ito IY. Bacterial profile in primary teeth with necrotic pulp and periapical lesions. Braz Dent J 2006;17:144-8.
22. Duggan JM, Sedgley CM. Biofilm formation of oral and endodontic Enterococcus faecalis. J Endod 2007;33:815-8.
23. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol 1999;37:1771-6.
24. Hoogenkamp MA, Crielaard W, ten Cate JM, Wever R, Hartog AF, Renirie R. Antimicrobial activity of vanadium chloroperoxidase on planktonic Streptococcus mutans cells and Streptococcus mutans biofilms. Caries Res 2009;43:334-8.
25. Vogel AI. A text-book of quantitative inorganic analysis, including elementary instrumental analysis. 3rd ed. London: Longmans; 1962.

26. Kara D, Luppens SB, Cate JM. Differences between single- and dual-species biofilms of Streptococcus mutans and Veillonella parvula in growth, acidogenicity and susceptibility to chlorhexidine. Eur J Oral Sci 2006;114:58-63.
27. Gabrielson J, Hart M, Jarelov A, Kuhn I, McKenzie D, Mollby R. Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates. J Microbiol Methods 2002;50:63-73.
28. Ito A, Taniuchi A, May T, Kawata K, Okabe S. Increased antibiotic resistance of Escherichia coli in mature biofilms. Appl Environ Microbiol 2009;75:4093-100.
29. Davies D. Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov 2003;2:114-22.