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Effect of laser-activated irrigation on biofilms in artificial root canals

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Abstract

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Aim To evaluate the antimicrobial effect of laser-activated irrigation (LAI) on biofilms formed in simulated root canals.

Methodology A dual-species biofilm of *Enterococcus faecalis* and *Streptococcus mutans* was grown in a resin root canal model. Biofilms were formed over 48 h and subsequently subjected to the following treatments, all executed for 20 s: syringe irrigation (SI) with a 27G needle, ultrasonically activated irrigation (UAI) with a size 20 Irrisafe file, and LAI with a 2940 nm Er:YAG laser (20 Hz, 50 μs, 20 or 40 mJ, conical fibre tip at two positions). Tests were performed with both sterile saline as well as NaOCl (2.5%) as the irrigant. Surviving bacteria were harvested and the number of CFU was determined by plate counting and compared across groups (ANOVA, $P \leq 0.05$).

Results Using saline as the irrigant, significant reductions in viable counts compared to untreated controls were observed for ultrasonically activated irrigation (0.52 log10 reduction) and for all laser-activated irrigation groups (>1 log10 reduction), but not for syringe irrigation (<0.25 log10 reduction). The reductions in the laser-activated irrigation groups were significantly greater than those of ultrasonically activated irrigation. With NaOCl as the irrigant, significant reductions (>2.2 log10 units) in the number of attached bacteria were observed for all treatment groups with no significant differences between laser-activated and ultrasonically activated irrigation.

Conclusions Within the limitations of this *in vitro* set-up, laser-activated irrigation removed more biofilm than ultrasonically activated irrigation when using saline as the irrigant, indicating greater physical biofilm removal. The use of NaOCl resulted in greater biofilm reduction with no significant differences between treatment groups.

Keywords: biofilm, disinfection, *Enterococcus faecalis*, erbium laser, PIPS, *Streptococcus mutans*.

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Introduction

The aim of root canal treatment is healing or prevention of apical periodontitis, a disease caused by infection of the root canal space (Kakehashi *et al.* 1965). Treatment involves mechanical enlargement of the main canals in combination with the use of chemicals to improve debridement and disinfection. Although this approach is effective in many cases, failures still occur (Siqueira 2001), and these are mainly caused by microorganisms remaining inside the root canal system (Sjögren *et al.* 1997, Molander *et al.* 1998).

Due to the complex three-dimensional microstructure of the root canal system, mechanical instrumentation does not prepare the entire canal surface (Peters *et al.* 2001), limiting its cleaning capacities. Therefore, adequate delivery and penetration of antimicrobial irrigant solutions within the three-dimensional microstructure is essential to allow

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efficient debridement and disinfection (Paque *et al.* 2011). Traditional root canal irrigation with a syringe-needle combination often fails in this respect (Wu & Wesselink 2001, Boutsioukis *et al.* 2009). A small but important number of bacteria may remain in the canals after conventional irrigation, even after apical enlargement (Falk & Sedgley 2005). Irrigant activation techniques have therefore been suggested to improve irrigant distribution in the canal system and increase irrigation effectiveness (Gu *et al.* 2009) and any such techniques or devices have been reported.

Ultrasonically activated irrigation (UAI) refers to the activation of irrigants through an oscillating non-cutting file placed in the prepared root canal. Its efficacy has been demonstrated in numerous studies (Sabins *et al.* 2003, Lee *et al.* 2004). Although cavitational effects have been observed with UAI (Jiang *et al.* 2011), its main cleaning action is attributed to acoustic microstreaming. Ultrasonic activation of the irrigant combined with the intermittent flush method produces a cumulative effect over three refreshment/activation cycles (van der Sluis *et al.* 2010).

Laser-activated irrigation (LAI) has been introduced as an alternative technique for activation of root canal irrigants. The cleaning mechanism of LAI depends on rapid fluid motion in the canal as a result of expansion and implosion of vapour bubbles at the fibre tip caused by the pulsed laser operation, a pure cavitation phenomenon (Blanken & Verdaasdonk 2007, Matsumoto *et al.* 2011). In addition, shock waves (Esenaliev *et al.* 1993) and secondary cavitation bubbles are believed to contribute to the cleaning action. It is very important that the wavelength of the laser used for LAI is in line with the resonance frequency of water to obtain an optimal absorption of the laser beam by the irrigant. The Er:YAG laser (Erbium, Yttrium-Aluminium-Garnet) emitting at 2940 nm and the Er,Cr:YSGG laser (Erbium; Chromium, Yttrium-Scandium-Gallium-Garnet) emitting at 2780 nm, are both suitable for LAI, since these wavelengths are close to the absorption peak of water (Wieliczka *et al.* 1989, Meire *et al.* 2014). The effect of LAI is further influenced by laser characteristics such as pulse length, pulse energy, pulse frequency, laser tip design and position.

LAI with erbium lasers has been shown *in vitro* to be more effective than conventional (syringe-based) and ultrasonically activated irrigation in terms of removal of debris from artificial (De Moor *et al.* 2009, 2010, de Groot *et al.* 2009, Arslan *et al.* 2014) or true (Lloyd *et al.* 2014) root canal irregularities as well as for smear layer removal in the apical part of the canal (George *et al.* 2008, Peeters & Suardita 2011, Divito *et al.* 2012, Guidotti *et al.* 2014).

Because the action of the irrigant induced by LAI is extremely turbulent, improved removal of microorganisms and biofilm from the canal system is to be expected by a purely physical effect. In addition, a chemical effect seems to play a role as well. Macedo *et al.* (2010) found an increased reaction rate of NaOCl upon activation by a pulsed erbium laser. The effect of the laser was stronger than that of UAI in this respect.

Several studies have investigated the antimicrobial effect of LAI. In terms of microbiological reduction, Peters *et al.* (2011) noticed that LAI generated more negative bacterial samples than ultrasonic irrigation of 6% NaOCl in root canals with an *ex vivo*-grown biofilm. Ordinola-Zapata *et al.* (2014), using a similar setup, observed significant better biofilm removal from biofilm-covered dentine blocks with LAI. On the contrary, Pedulla *et al.* (2012) found no significant difference in disinfection between conventional syringe irrigation and laser activation of 5% NaOCl in artificially contaminated root canals. Christo *et al.* (2015), using an Er;Cr:YSGG laser, found no better disinfection with LAI over syringe irrigation in an infected tooth model.

It is difficult to synthetize the results of the above-mentioned studies because of differences in methodology. For example, the biofilm model, the type of irrigant (saline, NaOCl), its concentration (between 1 and 6% NaOCl), the laser wavelength (2780 vs. 2940 nm), laser parameters (pulse energy, pulse frequency, position of fibre tip, irradiation time) vary amongst these studies.

By using NaOCl, a disinfectant, as an irrigant, the intracanal bacterial load is reduced both in a chemical and physical way. Because the action of the irrigant induced by LAI is extremely turbulent, it is interesting to know the purely physical effect of LAI on the removal of microorganisms. This is possible by using an irrigant that is not antimicrobial. It is hypothesized that the turbulent action of the irrigant induced by LAI results in better biofilm removal than traditional (syringe-based) and ultrasonically activated irrigation.

The purpose of this *in vitro* study was to evaluate the antimicrobial effect (physical as well as chemical) of UAI and different LAI protocols on intracanal biofilms.

Materials and methods

Root canal model

A resin root canal model was used to grow the biofilms (Fig. 1). The model is made up of five simulated root canals, each with a coronal reservoir resembling a pulp chamber. The model consists of two halves with the section through the centre of the canals. Both halves can be readjusted by means of a pin and hole guidance. The artificial root canals are 15 mm in length, with a taper of 6% and an apical diameter of 400 μm. The apical terminus of each canal was sealed. The models were sterilized in sodium hypochlorite 2.5% for 30 min and ethanol 70% for another 30 s. The two halves were reassembled and fixed with a clip after application of a silicone gel (Dow Corning High Vacuum Grease, Dow Corning Corporation, Midland, MI, USA) on the contact area in order to prevent fluid leakage.

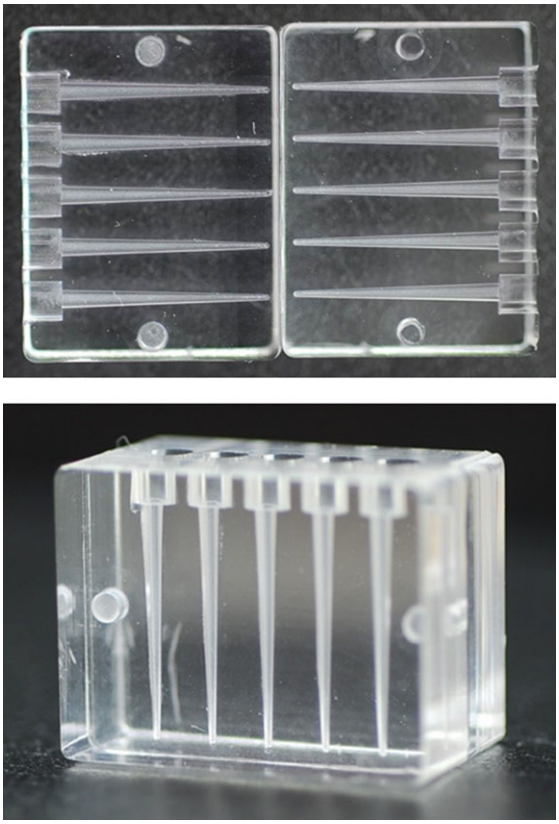

Figure 1 The resin root canal model.

Dual-species biofilm

The test species were *Enterococcus faecalis* (strain ATCC 10541) and *Streptococcus mutans* (strain LMG 14558). The bacteria were taken from a frozen stock culture, streaked onto brain–heart infusion (BHI) agar plates, and cultivated for 24 h at 37 °C. Cultures were conserved on BHIA at 4 °C and subcultured on a regular basis. Prior to each experiment, single colonies were inoculated into BHI broth and grown overnight at 37 °C. Cell suspensions were adjusted spectrophotometrically to an optical density (OD; 600 nm) of 0.2 to ensure equal bacterial inocula for each experiment. These suspensions were further diluted 1/1000 and 1/10.000 for *S. mutans* and *E. faecalis* respectively. As a result, the number of bacteria was approximately 10^4 cells mL^{-1}. Each artificial root canal was inoculated with a 1 : 1 mixture of both cell suspensions. The canals were filled to the level of the canal entrance. The inoculated resin blocks were put in a plastic vessel and incubated in a humid environment at 37 °C.

Initially, incubation times of 48, 72 and 168 h (7 days) were tested. Based on these results, an incubation time of 48 h was used for the remainder of the experiments.

Test groups

After 48 h of incubation, the BHI suspension was aspirated with a 30 gauge endodontic needle placed at the canal terminus with the intention to remove nonattached cells. In order to confirm the presence of a biofilm, five samples were inoculated for 15 min with Live/Dead® fluorescent staining (BacLight™ Bacterial Viability Kit, Molecular Probes, Eugene, OR, USA). The canal wall was then visualized by fluorescence microscopy (magnification 40×). The canals and the coronal reservoir of the remainder blocks were then filled with irrigant, and the models were assigned to the different treatment groups as follows:

- The positive control group received no further treatment.
- In the syringe irrigation group, irrigation was performed using a 27 gauge endodontic needle placed 1 mm short of the working length. Each root canal was irrigated with 3 mL of the irrigant with a steady flow rate of 0.3 mL per second.
- In the UAI group, the irrigant was ultrasonically activated using a noncutting size 20 file (Irrisafe, Satelec Acteon, Mérignac, France) driven by an

ultrasonic device (Suprasson Pmax Newtron, Satelec) at 50% power for 20 s, 1 mm from the working length.

Irrigant activation in the next groups was accomplished by using an Er:YAG laser (AT Fidelis, Fotona, Ljubljana, Slovenia) with a wavelength of 2940 nm. The laser was equipped with a hand piece (R14-PIPS, Fotona) holding a conical 400-μm fibre tip, 14 mm in length (X-pulse 400/14). The air and water spray was turned off. In order to avoid depletion of the pulp chamber, irrigant was added during activation, ensuring the presence of irrigant in the pulp chamber at all times.

- In group LAI 1, a conical fibre tip (X-pulse 400/14) was held at the canal entrance and activated for 20 s. The pulse energy was 20 mJ, the frequency 20 Hz and the pulse length 50 μs.
- Laser-activated irrigation in group LAI 2 was performed as in group LAI 1, but the pulse energy was set at 40 mJ.
- In group LAI 3, LAI was performed as in group LAI 1, but the laser tip was held 6 mm from the working length.

All experiments were executed with both sterile saline (0.85% [w/v] NaCl) and NaOCl (2.5%) as the irrigant. At least six blocks per treatment group were tested per experimental run and at least two runs were carried out, yielding a total of at least 12 blocks (60 artificial root canals) per treatment group.

Quantification of surviving bacteria

After treatment, the irrigant was aspirated with a 30 gauge endodontic needle, with the intention to remove nonattached cells. Each block was then disassembled and both halves were put in 10 mL of sterile saline. In the groups treated with NaOCl as the irrigant, the resin blocks were put in 10 mL of 2.5% sodium thiosulfate to inactivate any remaining NaOCl. Remaining cells were detached by a combination of sonication and vortex mixing (three cycles of 30 s each). Serial tenfold dilutions were made (10^{-1} to 10^{-6}) in sterile saline of which 1 mL was plated (pour plate method) on both BHI agar (BHIA) and an *Enterococcus*-selective BHI agar (BHI agar with added NaOH resulting in a pH of 9.6). The latter had been developed and tested to allow growth of *E. faecalis* and prohibit *S. mutans* growth (data not shown). After incubation, colonies were counted and mean numbers of CFU (colony-forming units) in each group were calculated and compared.

Statistical analysis

CFU counts were logarithmically transformed to normalize the data prior to statistical analysis. Data analysis was performed using one-way ANOVA with *post hoc* Games-Howell test (SPSS Inc, Chicago, IL, USA). The level of significance was set at 0.05.

Results

Influence of incubation time on biofilm formation in the root canal model

Table 1 shows the number of recovered cells after incubation of the blocks for 48, 72 and 168 h (7 days). There was no significant difference in number of *E. faecalis* and *S. mutans* cells recovered at 72 h compared to 48 h ($P = 1.00$). However, significantly less cells were recovered after 168 h ($P \leq 0.024$). Based on these results, 48 h of incubation was chosen for subsequent experiments. Figure 2 shows representative images of the 48-h biofilm in the model. Both the canal wall and the canal terminus are shown. Cells with an intact membrane are stained green. The walls are covered by large amounts of viable bacteria, confirming the presence of the biofilm.

Saline irrigant groups

The mean number of recovered bacteria per block (total bacterial load, i.e. both species combined) for different activation regimens of sterile saline is displayed in Table 2. In the control group, at least 10^7 CFU were recovered per block, and *E. faecalis* was found in higher numbers (mean log CFU/block of 7.34) than *S. mutans* (mean log CFU/block of 5.12). This was observed in all the treatment groups (data not shown).

Compared to the untreated controls, significantly less bacteria ($P \leq 0.001$) were observed in the UAI

Table 1 Number of recovered bacteria per resin block for different incubation times

		Mean log CFU ± SD		
Incubation time, h	*n*	*E. faecalis*	*S. mutans*	Total load
48	3	6.45 ± 0.23	7.34 ± 0.50	7.39 ± 0.47
72	3	6.15 ± 0.83	5.70 ± 0.73	6.28 ± 0.37
168	3	4.05 ± 0.39	3.93 ± 0.55	4.30 ± 0.77

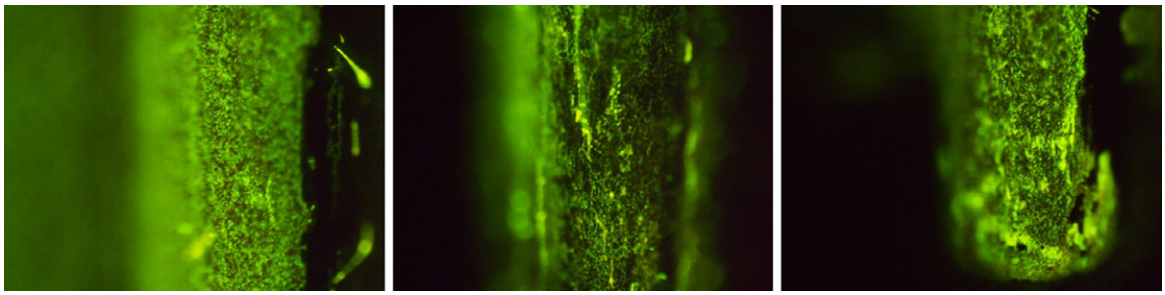

Figure 2 Representative fluorescence microscopy images of the biofilm on the canal wall in the untreated control samples.

Table 2 Mean number of recovered bacteria per block for different saline activation regimens

Treatment	*n*	Mean log CFU	Std dev	Log red
Untreated controls	66	7.51	0.25	0
Syringe irrigation	12	7.25	0.27	0.26
UAI	12	6.99	0.31	0.52
LAI 1	17	6.34	0.31	1.17
LAI 2	18	5.88	0.59	1.63
LAI 3	12	5.89	0.58	1.62

Mean log CFU, mean logarithmic number of colony-forming units; *n*, number of samples per group; std dev, standard deviation; log red, logarithmic reduction.

(0.52 $\log_{10}$ reduction) and in all LAI groups (>1 $\log_{10}$ reduction), but not in the syringe irrigation group (only 0.26 $\log_{10}$ reduction).

LAI groups 2 and 3 had the highest bacterial reductions (>1.6 $\log_{10}$ reduction). The results for both groups did not differ significantly from one another ($P > 0.05$), but did significantly differ from all the other treatment groups ($P \leq 0.033$). The number of recovered bacteria in group LAI 1 was significantly different from all other treatment groups ($P \leq 0.033$).

NaOCl irrigant groups

The mean number of recovered bacteria per block (total bacterial load) for different activation regimens of NaOCl is shown in Table 3. The use of NaOCl as the irrigant resulted in significant reductions in viable counts for all treatment groups (>2.2 $\log_{10}$ reductions, $P < 0.001$) (Table 3). The highest reductions were observed in the UAI and LAI 2 and 3 groups, with 3.35, 3.37 and 3.35 $\log_{10}$ reductions respectively. LAI Groups 2 and 3 resulted in significantly higher reductions in CFU per resin block than syringe irrigation ($P < 0.05$). No other significant differences between groups were observed.

Discussion

In the present study, a highly standardized setup was used to investigate the antimicrobial effect of different irrigant activation methods on a dual-species *E. faecalis*/*S. mutans* biofilm grown in artificial root canals. When saline was used as the irrigant, LAI yielded the greatest biofilm removal. With NaOCl as the irrigant however, greater reductions were obtained in all groups with no significant differences between the activation methods.

The main advantage of the *in vitro* root canal model developed is the high degree of standardization of experimental conditions due to exclusion of root canal anatomy, root canal preparation, and dentine composition as a variable which is the case when working with extracted (human) teeth. Also, the use of the model reduces working time as it is no longer necessary to collect, evaluate, prepare and sterilize extracted teeth. In this way, it is possible to execute more runs and to achieve larger test groups. Finally, the sectional halves allow exposure of the canals after treatment, enabling a more thorough method of removing surviving cells (through the combination of sonication and vortex mixing). This allows a better

Table 3 Mean number of recovered bacteria per block for different NaOCl activation regimens

Treatment	*n*	Mean log CFU	Std dev	Log red
Untreated controls	66	7.51	0.25	0
Syringe irrigation	18	5.29	1.08	2.22
UAI	10	4.16	1.25	3.35
LAI 1	11	4.61	0.87	2.90
LAI 2	12	4.14	1.37	3.37
LAI 3	12	4.16	1.28	3.35

Mean log CFU, mean logarithmic number of colony-forming units; *n*, number of samples per group; std dev, standard deviation; log red, logarithmic reduction.

sampling of remaining bacteria compared to the use of the paper point method of bacterial sampling as used in other studies (Peeters & Suardita 2011, Pedulla *et al.* 2012). Besides these advantages, this model also offers the possibility to study other variables such as canal dimensions, shape and curvature, the effect of grooves, the irrigation time and the use of intracanal medication.

Because the number of cells recovered after 72 h of incubation was not significantly different from the number obtained after 48 h of incubation, and significantly higher than that after 7 days, 48 h incubation time was chosen for *in vitro* biofilm formation. This duration of incubation might be rather short compared to other studies. For example, it has been proven by Shen *et al.* (2011) that bacteria in mature (3-week old) biofilms are more resistant to antimicrobial treatment than in young biofilms. In this respect, the biofilm in the present study is rather young and perhaps more susceptible to antimicrobial treatment. On the other hand, no consensus currently exists about appropriate setups and incubation times in order to develop a representative biofilm model, accentuating the importance of developing standardized intracanal microbial biofilm models for endodontic experiments. It is safe to say that in the present model a biofilm has been applied that consisted of two species and was grown into stationary phase, thus representing maximum cell numbers.

The total bacterial counts for *S. mutans* were always lower than those for *E. faecalis*. This was noticed in each treatment group. The lesser growth of *S. mutans* in the presence of *E. faecalis*, was also observed previously (Deng *et al.* 2009).

Sterile water was used as the irrigant in the first part of the study. No antibacterial agent was used because this part was designed to assess only the mechanical effects of irrigant activation. Reductions in bacterial counts after treatment with saline irrigant were low (between 0.26 and 1.63 log 10 reduction) and syringe irrigation did not result in a significant reduction compared to the untreated controls. The reduction in the UAI group was significant but low (0.5 log red). The highest reductions were obtained in the LAI groups (>1 log red). These results indicate the stronger mechanical effect of LAI compared to UAI and SI, which is attributed to the extremely turbulent action of the irrigant induced by pulsed erbium lasers. The reductions obtained with saline in the present study are in line with those in the study of Pedulla *et al.* (2012), albeit slightly higher. They found 0.06 and 0.57 $\log_{10}$ reductions in the SI and LAI (20 mJ) group respectively.

Interestingly, a higher pulse energy (40 instead of 20 mJ) and a deeper position of the fibre tip inside the canal (6 mm from working length instead of at the canal entrance), significantly increased the bacterial reduction. The effect of higher pulse energy can probably be explained by the resulting higher peak powers which yield larger primary cavitation bubbles at the fibre tip and hence greater liquid displacement and more violent shock waves. In LAI, the most intense fluid motions are located near the fibre tip (de Groot *et al.* 2009), which explains why placement of the fibre tip deeper inside the canal resulted in better biofilm removal than placement of the tip in the canal entrance.

These results are also in accordance with findings from other studies. Deleu *et al.* (2015) found that holding a flat fibre tip inside the root canal resulted in better debris removal from root canal irregularities than holding a conical fibre tip at the canal entrance.

However, besides antimicrobial efficiency, safety is another and equally important aspect of irrigation, and extrusion of irritating solutions beyond the apical foramen should be avoided at all times. One could argue that higher pulse energies and more apical fibre positioning pose higher risk of irrigant extrusion. Although George & Walsh (2008) found similar extrusion rates when the fibre tip was placed at 5 or 10 mm from the working length, and Arslan *et al.* (2015) found no significant difference in extrusion between pulses of 20 or 30 mJ, care has to be taken with these *in vitro* setups as these extrusion models are far from the clinical reality.

When NaOCl was used as the irrigant, reductions were greater for all treatment groups. Syringe irrigation now significantly decreased the intracanal load compared to the untreated controls. The only significant differences that remained between treatment groups were between syringe irrigation and LAI groups 2 and 3. Thus, in the present model, the chemical action of NaOCl seemed to be more important than the physical effects of the irrigant activation, thereby levelling off the differences between treatment groups. Also, the laser treatment did not seem to significantly increase the chemical action of NaOCl.

Whilst the bacterial reductions with NaOCl were far greater than those with saline, a substantial portion of the microbial load remained after NaOCl irrigation and activation. A possible explanation is the

limited contact time between NaOCl and the canal walls in the present study (less than 30 s).

Whilst the resin root canal model ensured optimal standardization of experimental conditions, possible drawbacks of the present model include the absence of dentine as the substratum for biofilm development, which may result in an altered adhesion of the biofilm to the surface. Also, the smooth resin walls of the canals, their perfectly round cross-sectional shape and straight course are different from the clinical situation and might impact on the fluid dynamics. In the absence of dentine, the effect of NaOCl in the present setup could also have been overestimated since in the root canal, NaOCl reacts with collagen, fluid and biomass within the dentine yielding less available chlorine for disinfection (Macedo *et al.* 2010). Another issue of the present model is the potential bacterial growth in the contact area of the resin blocks. Bacterial growth at the interface could not be excluded and this could be an explanation for the small differences in number of recovered bacteria between the treatment groups. Therefore, care should be taken to directly extrapolate the present results to the case of complicated natural root canal anatomy situations.

Conclusion

Within the limitations of this *in vitro* set-up, laser-activated irrigation removed more biofilm than ultrasonically activated irrigation when using saline as the irrigant, indicating greater physical biofilm removal. The use of NaOCl resulted in greater biofilm reduction with no significant differences between treatment groups.

Conflict of interest

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

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