Insights into the bacterial and fungal ecology of endodontic infections
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Citation for published version (APA):
Persoon, I. F. (2016). Insights into the bacterial and fungal ecology of endodontic infections

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Effect of vanadium chloroperoxidase on Enterococcus faecalis biofilms

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Journal of Endodontics 2012; 38: 72-74

Garganta del Diablo near Puerto Iguazú, Argentina.

Half of the flow of Iguazú river falls into the waterfall Devil’s Throat. Similar to the immense thunder of water, VCPO should rigorously clean the root canal system.
ABSTRACT

AIM
The aim of this study was to explore the antimicrobial effect of vanadium chloroperoxidase (VCPO) reaction products on *Enterococcus faecalis* biofilms of four different strains.

METHODOLOGY
Twenty-four-hour biofilms of *E. faecalis* strains V583, ER5/1, E2 and OS-16 were incubated in mixtures with VCPO, halide (either bromide or chloride) and hydrogen peroxide. The antibacterial efficacy was assessed by colony-forming unit counts.

RESULTS
The VCPO reaction products had a similar efficacy in reducing the viability of the four strains of *E. faecalis* (94%, range 87 - 100%). Bromide as the halogen of choice was more effective on *E. faecalis* strains E2 and OS-16, as compared to chloride (Mann-Whitney U test; *P* < 0.05). Despite different quantities of produced biofilms by the four strains, VCPO treatment was similarly effective towards all strains (Kruskal-Wallis test; *P* < 0.05).

CONCLUSION
VCPO treatment results in an antimicrobial effect towards *in vitro E. faecalis* biofilms and might provide an addition to current endodontic treatment, possibly as an antimicrobial dressing.
INTRODUCTION
Apical periodontitis is caused by an infection of the root canal system, and eradication of the microbiota is required for treatment (Kakehashi et al. 1965). Current techniques of chemomechanical treatment have proven to be insufficient in achieving this goal (Kirkevang 2011). One of the virulence factors contributing to the persistence of these bacteria is their ability to form biofilms, which results in increased resistance to endodontic irrigants (Chávez de Paz 2007). Another limiting factor of current endodontic treatment procedures is their inability to reach the entire elaborate and complex root canal system (de Pablo et al. 2010).

*Enterococcus faecalis* is a bacterium frequently found in endodontic infections, especially in retreatment cases (Sundqvist & Figdor 2003). Its persistence, despite treatment and restrained conditions, can be attributed to several virulence factors, such as its ability to grow in alkaline environments and its antibiotic resistance (Sundqvist & Figdor 2003). Most endodontic research focuses on the eradication of this bacterium (Chávez de Paz 2007); therefore, *E. faecalis* was used in the current study.

The reaction products of the enzyme vanadium chloroperoxidase (VCPO) from the fungus *Curvularia inaequalis* have been shown to possess antimicrobial activity against both planktonic cells and biofilms of oral pathogenic bacteria (Hoogenkamp et al. 2009). Their effectiveness towards endodontic pathogens has, thus far, not been analysed. Vanadium haloperoxidases are extremely stable enzymes and display a high stability towards potential denaturing agents, such as organic solvents, singlet oxygen and high temperatures (Van Schijndel et al. 1994; Renirie et al. 2003). In a slightly acidic environment, VCPO catalyses the oxidation of halides, such as chloride and bromide, in the presence of low concentrations of hydrogen peroxide (H$_2$O$_2$), into hypohalous acid (equation 1). Through a secondary reaction, occurring spontaneously, singlet oxygen is formed (equation 2). Both hypohalous acid and singlet oxygen have been shown to possess strong antimicrobial properties (Pellieux et al. 2000).

\[
\begin{align*}
X^- + H_2O_2 + H^+ &\rightarrow HOX + H_2O & \text{(equation 1)} \\
HOX + H_2O_2 &\rightarrow O_2 + H_2O + X^- + H^+ & \text{(equation 2)}
\end{align*}
\]

\(X = \text{Cl or Br}\)

The high stability of the enzyme in combination with the continuous production of antimicrobial products allows for a broad range of applications. These might include hard surface antifouling, surface disinfection, cleansing of medical instruments and contact lenses (Barnett et al. 1995; Hansen et al. 2003) and
as a potential antimicrobial dressing for degradation of endodontic biofilms. At the moment, the limited shelf life restricts the use of hypohalous acid (Zehnder 2006). Prolonged, continuous, and local production of strong antimicrobials by VCPO could overcome this limitation, thereby extending the current range of inactivation of microorganisms in the root canal system.

Therefore, the aim of the present study was to explore the antimicrobial efficacy of the VCPO reaction products on *E. faecalis* biofilms of four different strains.

**METHODOLOGY**

*Bacterial strains and enzyme preparation*

Four different *E. faecalis* strains were used in this study. Strain V583 was isolated in a hospital infection in 1989 and its genome has been sequenced (Sahm et al. 1989). Strains E2 (Sedgley et al. 2004) and OS-16 (Sedgley et al. 2005) are oral isolates. Strain ER5/1 (Johnson et al. 2006) has been isolated from an endodontic retreatment case. All strains were routinely cultured on Brain-Heart Infusion (BHI) medium supplemented with 1.5% Agar (BD, Sparks, MD) at 37°C under anaerobic conditions (10% H₂, 10% CO₂ in N₂). Biofilms were grown in modified semi-defined biofilm medium (BM) (Deng et al. 2009).

Vanadium chloroperoxidase from the fungus *C. inaequalis* was prepared as previously described (Hasan et al. 2006).

*Biofilm treatment*

Biofilms were grown on polystyrene pegs as previously described (Hoogenkamp et al. 2009). In brief, cultures of all four strains, inoculated from freezer stocks, were grown overnight at 37°C under anaerobic conditions in BM supplemented with 0.36% glucose. Overnight cultures were adjusted to a final optical density of OD₆₂₀ = 0.02 in BM with 0.2% sucrose, to enhance biofilm production. Of each inoculum 200 μL per well was distributed into a 96-well microtitre plate (NUNC, Roskilde, Denmark). An Immuno TSP PolySorp lid with 96 corresponding polystyrene pegs (NUNC) was placed on the plate for attachment of the biofilm. Biofilms were grown for 24 hours under anaerobic conditions at 37°C, with refreshment of BM supplemented with 0.2% sucrose after 8 hours. The biofilms were washed three times with buffered peptone water (BPW) (Oxoid Ltd, Basingstoke, UK) to remove nonadherent bacteria.

Biofilms were treated as described by Hoogenkamp et al. (2009). In brief, the pegs with biofilms were inserted into a new 96-well microtitre plate with 180 μL incubation of 50 mM citrate buffer pH 5.5 (Merck, Darmstadt, Germany), 1 μM VCPO and with either 25 mM sodium chloride (NaOCl) or 1 mM potassium bromide (Sigma-
Incubations without VCPO served as negative controls. The reaction was initiated by addition of 10 mM hydrogen peroxide (Merck) per well. An incubation of 2% NaOCl, which is equivalent to a concentration of 260 mM, served as a positive control. The plate was incubated for five minutes, after which the biofilm was washed three times with BPW to remove any active agents.

The pegs with the biofilm were removed from the lid and put into 1 mL cysteine peptone water (CPW) (Hoogenkamp et al. 2009). Samples were vortexed and sonicated for 30 times, 1 s, 40 Hz (VC130 Ultrasonic processor, Sonics & Materials Inc., Newtown, CT), then serially diluted in CPW and spiral plated (EddyJet, IUL instruments, Barcelona, Spain) onto BHI agar plates. The plates were incubated under anaerobic conditions at 37°C for 48 hours, after which colonies were counted. The experiment was performed four times in triplicate (N = 12).

**Statistical analysis**

Data were analysed using SPSS version 18.0 (Chicago, IL). Statistical analyses were performed on log10 converted data. Data were not normally distributed and, therefore, Mann-Whitney tests and Kruskal-Wallis tests were used to compare the different groups. Post-hoc analyses were performed using Tukey’s tests. The level of significance was set at α = 0.05.

**RESULTS**

The VCPO reaction products showed antimicrobial activity on all four tested strains of *E. faecalis* (Mann-Whitney U test; *P* < 0.05). Figure 6.1 shows the percentage of
bacterial inactivation per strain and halide. Bromide as a halogen source in addition to VCPO was significantly more effective in reducing colony-forming units (CFU) counts, compared with chloride (Mann-Whitney U test; \( P < 0.05 \)). When the four different \( E. \) faecalis strains were compared, the amounts of biofilm generated by these strains significantly differed (Kruskal-Wallis test; \( P < 0.05 \)) by a factor 5 to 9. Strain V583 continuously produced the least biofilm (Tukey honestly significant difference; \( P < 0.05 \)). All strains were inactivated with a similar effectivity (Kruskal-Wallis test; \( P < 0.05 \)). The control with 2\% NaOCl showed a 100\% bacterial inactivation, which is similar to the effect of VCPO.

DISCUSSION
The aim of this study was to evaluate the antimicrobial effect of VCPO reaction products on \( E. \) faecalis. All four strains of \( E. \) faecalis showed reduced CFU counts after VCPO treatment (94\%, range 87 - 100\%). Previous work on \textit{Streptococcus mutans} biofilms by using VCPO (Hoogenkamp \textit{et al.} 2009) also showed a significant bactericidal effect. Although biofilms show greater resistance towards treatment than planktonic cultures (Chávez de Paz 2007), VCPO reaction products were significantly effective in inactivating \( E. \) faecalis biofilms.

Current endodontic treatments mostly use rinsing schemes, often with NaOCl, allowing for only a limited exposure time on the biofilm (Zehnder 2006) and not penetrating all the complex irregularities of the root canal system (de Pablo \textit{et al.} 2010). Although a 2\% NaOCl solution has been shown to possess great antimicrobial properties, the \textit{in vivo} effects are expected to be lessened through inactivation by dentin (Haapasalo \textit{et al.} 2000) and short exposure time (Macedo \textit{et al.} 2010). Our controls are thus expected to perform less effectively in a short rinsing scheme in the elaborate root canal complex during current treatment methods. A possible alternative and elongated treatment could be the application of VCPO as an intracanal interappointment dressing, because the enzyme shows high stability and strong antimicrobial activity. Because halides are not consumed during the reaction, only a small quantity would be necessary for the reaction. They can be added to the dressing, but as an additional benefit, are also present naturally in the human body. Chloride is present in both saliva and serum at concentrations of 16 mM and 32 mM, respectively (Rehak \textit{et al.} 2000), and bromide at concentrations of 0.17 mM and 0.06 mM, respectively (Michigami \textit{et al.} 1989). Oral bacteria are able to produce hydrogen peroxide (Carlsson \textit{et al.} 1983), implying that all the necessary substrates are present in substantial concentrations for VCPO to catalyse the continuous production of antimicrobials. These substrates and the VCPO reaction products
do not damage the enzyme; they have shown up to 25,000 turnovers (Renirie et al. 2003). Because of the high stability of the enzyme (Van Schijndel et al. 1994; Renirie et al. 2003) and continuous presence of substrate, prolonged antimicrobial activity is shown. This increased exposure time might result in inactivation of the biofilm deep into the irregularities of the root canal system.

The options of simply using hypohalous acid, one of the VCPO reaction products, as a commercial endodontic treatment product are limited. Hypohalous acid has a very short shelf-life because it quickly disintegrates and loses its antimicrobial properties (Zehnder 2006). Local intracanal production, however, provides fresh and active hypohalous acid continuously. The produced hypohalous acid has a more potent effect on bacteria than a similar hypohalite, such as hypochlorite (Bloomfield & Miles 1979). Also, complementary antimicrobial activity could be provided by the additional product of the VCPO reaction, singlet oxygen (Pellieux et al. 2000).

The high stability of the enzyme might pose a possible limitation to its use, with regard to biocompatibility. Contact between the continuously supplied VCPO reaction products and the periapical tissues is possible. Although more specific research has to be done concerning the biocompatibility of VCPO, a similar reaction takes place all over the human body. The myeloperoxidase enzyme in neutrophils and gingival fluids and the lactoperoxidase enzyme in saliva (Kersten et al. 1981) catalyse the formation of hypohalous acid and singlet oxygen for inactivation of invading bacteria. However, the reaction products and their substrate inhibit the enzyme, thereby self-containing the reaction. Most conventional endodontic treatments use hypochlorite, which can create serious injuries on intrusion of the periapical tissues. Hypochlorous acid has proven to be less harmful when compared with hypochlorite (Wang et al. 2007), although more detailed research has to be done regarding the actual effect on contact with and incidental injection into the periapical tissues. Current available methods to inactivate highly stable enzymes such as VCPO are too radical to be applicable within the human body (Van Schijndel et al. 1994; Messerschmidt & Wever 1996). However, the activity of the enzyme might decrease slowly in time because of exchange of the vanadate prosthetic group with free phosphate (Tanaka & Wever 2004). Complete consumption of one of the substrates, such as catalase converting the available hydrogen peroxide, could also confine the extent of the reaction. Another possibility to end the process is removal of the enzyme from the root canal system, although similar difficulties would be faced as on removal of a calcium hydroxide dressing. Complete removal of the enzyme from the root canal system is complex and traces of the enzyme still present might have adverse effects on obturation. These possibly include future leakage and outgrowth.
of remaining bacteria or recolonization (Hosoya et al. 2004). Thus, potential means to either inactivate the VCPO enzyme or contain the enzymatic reaction products remain to be studied.

CONCLUSION
The reaction products of VCPO have a significant antimicrobial effect on in vitro E faecalis biofilms and might provide an addition to current endodontic treatment to achieve complete inactivation of the biofilm in the root canal system. Future research should focus on the effect of VCPO on biofilms more similar to the in vivo situation, i.e. multi-species biofilms, because true endodontic biofilms are always composed of many more (Chávez de Paz 2007). Also, the possibility and safety of such an application in the clinical setting should be explored.

ACKNOWLEDGEMENTS
The authors would like to thank dr. C.M. Sedgley for providing the E. faecalis clinical strains. The authors deny any conflicts of interest related to this study.

AUTHOR CONTRIBUTIONS
Conceived and designed the study: IFP, MAH, PRW, WC. Performed the study: IFP, MAH, AB, AFH, RW. Analysed the data: IFP, MAH. Drafted the manuscript: IFP, MAH. Critically revised the manuscript: AB, PRW, AFH, RW, WC. All authors accepted the final version of the manuscript.