
Survival of *Enterococcus faecalis* in root canals *ex vivo*

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Abstract

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Aim The hypotheses tested in this study were that: (i) *Enterococcus faecalis* can survive long-term entombment in root filled teeth without additional nutrients, (ii) initial cell density influences the survival of *E. faecalis* in instrumented root canals and (iii) gelatinase-production capacity influences the survival of *E. faecalis* in root canals.

Methodology The root canals of 150 extracted single canal teeth were instrumented to apical size 60 and divided into six groups of 25. Within each group 10 canals were inoculated with either gelatinase-producing *E. faecalis* OG1-S and the other 10 with its gelatinase-defective mutant *E. faecalis* OG1-X. Five canals per group were kept as uninoculated controls. The root canals in groups 1 and 2 were inoculated with 10^6 bacteria, incubated for 48 h at 37 °C then filled with gutta-percha and zinc-oxide eugenol sealer. Root canals were inoculated with 10^6 , 10^5 , 10^4 and 10^3 bacteria in groups 3–6, respectively, and left unfilled. All teeth were sealed coronally with glass-ionomer

cement. After 6- (groups 1, 3–6) and 12-month (group 2) incubation at 37 °C in 100% humidity, root fragments were analysed for presence of *E. faecalis*, using culture, polymerase chain reaction and histological methods.

Results Viable *E. faecalis* was recovered from all root filled teeth and from 95–100% of unfilled inoculated teeth. Initial cell density and gelatinase production did not influence the recovery of viable *E. faecalis* ($P > 0.05$; chi-square test). *Enterococcus faecalis* 16S rRNA gene products were present in all inoculated teeth and absent in all noninoculated controls. Dentinal tubule infection was evident under light microscopy in sections from inoculated teeth after 48-h, 6- and 12-month incubation.

Conclusions *Enterococcus faecalis* inoculated into root canals maintained viability for 12-months *ex vivo*. The clinical implications are that viable *E. faecalis* entombed at the time of root filling could provide a long-term nidus for subsequent infection.

Keywords: *Enterococcus faecalis*, *in vitro*, root canal, root filling, survival.

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Introduction

Studies on primary root canal treatment have indicated that a negative culture at the time of filling is associated with a better prognosis (Byström *et al.* 1987, Sjögren *et al.* 1997). Sjögren *et al.* (1997) reported that the

presence of bacteria at filling was associated with the presence of apical periodontitis 5 years after treatment in 32% of the cases. Persistent radiolucent lesions have been associated with bacteria in root filled, asymptomatic teeth with periapical lesions (Nair *et al.* 1990). However, the difficulty in rendering canals bacteria-free *in vivo* has been well-documented, regardless of instrumentation method and irrigation regimen (Byström & Sundqvist 1985, Card *et al.* 2002).

Enterococcus faecalis has long been implicated in persistent root canal infections (Engström 1964) and more recently has been identified as the species most

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commonly recovered from root canals of teeth with post-treatment disease (Sundqvist et al. 1998, Siqueira & Rôças 2004). Three of nine cases from which *E. faecalis* was recovered at the time of retreatment failed to heal after 5 years (Sundqvist et al. 1998). The recovery of *E. faecalis* from failed previously filled root canals does not seem to be related to the use of a particular root filling material (Peciulienė et al. 2001). Enterococci might be selected in root canals undergoing standard endodontic treatment because of low sensitivity to antimicrobial agents (Dahlén et al. 2000) or their ability to inactivate antimicrobial agents (Portenier et al. 2002).

Siqueira Jr (2001) speculated that bacteria entombed by the root filling material usually die, but that some entombed species 'probably survive for relatively long periods', deriving nutrients from tissue remnants. The ability of *E. faecalis* to survive 'entombed' in root canals over an extended period has never been measured. This study tested the hypotheses that: (i) *E. faecalis* can survive 'entombment' in filled root canals, (ii) initial cell density influences the survival of *E. faecalis* in instrumented root canals and (iii) gelatinase-production capacity influences the survival of *E. faecalis* in root canals.

Materials and methods

Microorganisms

Two isogenic *E. faecalis* strains were used in this study: the gelatinase-producing, streptomycin resistant, *E. faecalis* OG1-S and its reduced gelatinase-producing mutant derivative, *E. faecalis* OG1-X.

Enterococcus faecalis strains were stored at -80 °C. When required, each strain was inoculated onto Todd-Hewitt broth (THB) (Difco, Becton Dickinson and Company, Sparks, MD, USA) supplemented with 1.5% agar and incubated aerobically at 37 °C for 24-48 h. One colony-forming unit was transferred to

5 mL THB and grown in aerobic incubation for 24 h at 37 °C. Bacterial suspensions (50 µL) were transferred to 5 mL fresh THB and grown to late exponential phase at which point cells were harvested. Cultures were centrifuged at 1500 g for 10 min. Deposits were washed twice in phosphate-buffered saline (0.1 mol L⁻¹, pH 7.3) and reconstituted in THB to a final concentration of approximately 1 × 10⁹ microorganisms per mL, corresponding to OD 0.75 at 660 nm (Spectronic 20D+; Thermo Electron Corporation, Pittsburgh, PA, USA). Serial dilutions (1 : 10) corresponding to 10⁸, 10⁷ and 10⁶ cells mL⁻¹ were prepared in THB. Calibration between viable counts and suspension optical density was confirmed by spiral plating serial dilutions to THB agar in triplicate and enumerating after 48 h aerobic incubation at 37 °C.

Selection and instrumentation of teeth

Permanent anterior teeth (n = 152) with single straight roots, clinically intact crowns, no restorations, no cracks and no caries were selected from the collection of extracted teeth stored in 50% glycerine/50% ethyl alcohol at the School of Dentistry, University of Michigan, Ann Arbor, MI, USA. Teeth were autoclaved at 121 °C for 15 min at 26 psi and stored at 25 °C in 100% humidity until use. Two teeth were used for histological observations described below. The remaining 150 teeth were divided into six groups (Table 1). Root canals were instrumented to an apical size 60 at working length (WL) as previously described (Sedgley et al. 2004a). Access openings were prepared using tungsten carbide burs in a high-speed handpiece with constant water spray. WL was set at 1 mm short of the apical foramen by visual inspection of a size 15 stainless steel K-file extending beyond the apical foramen. Canals were instrumented using a crown-down method and nickel-titanium (Ni-Ti) rotary and hand instruments (Dentsply; Tulsa Dental Products,

Table 1 Study design

	Filled canals		Unfilled canals			
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Incubation period (month):	6	12	6	6	6	6
Inoculum (cells per 15 µL):	10 ⁶ cells	10 ⁶ cells	10 ⁶ cells	10 ⁵ cells	10 ⁴ cells	10 ³ cells
Experimental						
<i>Enterococcus faecalis</i> OG1-S	10	10	10	10	10	10
<i>Enterococcus faecalis</i> OG1-X	10	10	10	10	10	10
Sterility controls						
No inoculum	5	5	5	5	5	5

Tulsa, TN, USA). Orifice openers and ProFile .06 Series 29 Rotary Ni-Ti files were used to prepare the coronal two-thirds of the root canal and Profile .04 Ni-Ti hand files to prepare the apical one-third to an apical size 60. Copious irrigation with 5.25% sodium hypochlorite was used between instruments. A 0.012 mm diameter round diamond bur in a high-speed handpiece was used to make 0.5 mm deep grooves on the mesial and distal aspects of each tooth, avoiding the apical 2 mm of narrow roots in order to not penetrate into the root canal; the purpose of this step was to facilitate subsequent splitting of the tooth at the completion of the incubation period.

For each tooth in groups 1 and 2, a master gutta-percha cone (Greater Taper Gutta-Percha; Analytic Endodontics, Glendora, CA, USA) was custom-fitted to WL and indented at the level of the coronal reference point. Each master gutta-percha cone was transferred to a labelled, empty 0.6 mL polymerase chain reaction (PCR) tube with attached cap (Dot Scientific Inc., Burton, MI, USA). In addition, nonstandardized gutta-percha cones (Fine-Fine; Kerr, Romulus, MI, USA), for use as accessory cones, were sealed in PCR tubes. Gutta-percha cones were sterilized by γ -radiation at the Ford Nuclear Reactor Phoenix Memorial Laboratory (University of Michigan).

The smear layer was removed from canal walls by ultrasonic treatment for 4 min each in 17% EDTA and 5.25% NaOCl (Haapasalo & Ørstavik 1987) after which canals were copiously rinsed with distilled water and dried with paper points. Root apices were coated with nail varnish to seal the apical foramen. Teeth were inserted with crown exposed into individual compartments in polypropylene boxes and autoclaved at 121 °C for 15 min at 26 psi, then aseptically stored in 100% humidity at 25 °C until use.

Inoculation and filling of root canals (groups 1 and 2)

All following procedures were performed under strictly aseptic conditions. Immediately before inoculation with bacteria, canals were dried with sterile paper points (Kerr). The root canals of teeth in groups 1 and 2 were inoculated with 10^6 cells in 15 μ L THB and aerobically incubated at 37 °C for 48 h to allow penetration of *E. faecalis* into root canal dentine. Immediately prior to filling, canals were dried with paper points (Kerr) placed to WL. Canals were filled to WL by an endodontist using cold lateral compaction with gutta-percha and previously unopened sealer (Roth-801;

Roth International Ltd, Chicago, IL, USA). The sealer was mixed according to the manufacturer's instructions on a sterile slab using a sterile spatula. The sealer was introduced into the canal to WL using a size 60 stainless steel K-file that was rotated counterclockwise as it was withdrawn. The apical half of each prefitted custom gutta-percha cone was coated lightly with sealer and placed to the WL. Cold lateral compaction was performed using a sterile D11T hand spreader (Kerr) and nonstandardized gutta-percha cones. Excess coronal gutta-percha was seared off at the level of the cemento-enamel junction using the System B heat source (SybronEndo, Orange, CA, USA) at 300 °C.

Inoculation of root canals (groups 3–6)

Each root canal was inoculated with 15 μ L of microbial suspension. Canals in groups 3–6 were inoculated with 10^6 , 10^5 , 10^4 and 10^3 cells, respectively (Table 1).

Restoration of access cavities

Access cavities were sealed with glass-ionomer cement (Ketac-Fil Plus; 3M-ESPE, AG, Seefeld, Germany) immediately following filling for groups 1 and 2, and after inoculation for teeth in groups 3–6. This material was selected based on previous reports of its sealability over long periods of time (Barthel *et al.* 1999). *In vitro* agar diffusion and broth dilution studies showed that Ketac-Fil Plus did not have bactericidal activity against *E. faecalis* OG1-S and *E. faecalis* OG1-X over concentrations ranging from 10^1 to 10^9 cells mL⁻¹ (data not shown).

Incubation of teeth

Each tooth was transferred to an empty labelled screw-top 2 mL sterile cryogenic vial (Corning Costar Corporation, Cambridge, MA, USA) and the cap replaced loosely in order to allow maintenance of humidity. Vials were placed in individual compartments in sterile polypropylene boxes containing sterile water at their base. Boxes were sealed hermetically and placed in an incubator at 37 °C under aerobic conditions.

Splitting teeth and assessment of viability of microorganism in teeth

At the end of each incubation period, each tooth was aseptically transferred from its individual vial to the base of a sterile foil-covered Teflon beaker (Nalgene

Labware, Rochester, NY, USA). Teeth were aseptically split into fragments using sterile long-handled end-cutter pliers (Channellock Inc., Meadville, PA, USA). Care was taken not to introduce any contaminants by keeping the beaker covered during splitting. All inoculated and uninoculated sterility control teeth were split using this method. Tooth fragments were aseptically transferred for distribution among (i) two 10 mL test tubes containing 5 mL THB broth, (ii) two 10 mL test tubes containing 4 mL THB broth supplemented with 1 mL human serum (Sigma-Aldrich, St Louis, MO, USA) and (iii) bile esculin agar (BEA; Difco). The contents of each tube were vortexed very vigorously to enable displacement of cells from tooth structure into suspension. Pairs of test tubes containing tooth fragments in THB alone and in THB/serum were incubated at 37 °C for up to 72 h under aerobic and anaerobic conditions (candle jar). Growth in broth was monitored by measuring optical density at 660 nm. BEA agar was incubated aerobically at 37 °C. After 72 h each tooth was scored as positive or negative for evidence of viable *E. faecalis* as determined by growth under any condition. Confirmation of *E. faecalis* OG1-S and OG1-X was made by culturing a loopful of suspension from each positive broth culture onto THB agar supplemented with 500 µg mL⁻¹ streptomycin, BEA and 3% gelatin agar which were aerobically incubated at 37 °C for up to 72 h.

Presence of *E. faecalis* by 16S rRNA gene using polymerase chain reaction

For each tooth sample in groups 1 and 2, the presence of *E. faecalis* in 1.5 mL vortexed suspensions of tooth fragments in THB was assessed by 16S rRNA gene product detection using end-point PCR and by investigators blinded to the culture results. These steps were not to confirm viability but to determine if the method had allowed displacement of cellular material into the suspension and to check sterility controls.

Methods have been previously described for total DNA isolation, development of universal bacteria and *E. faecalis*-specific 16S rRNA gene primer pairs and analyses of PCR products (Sedgley et al. 2005a). PCR amplifications were prepared in a 30 µL final reaction volume with the following: 100 ng total DNA template; 6 pmol of each primer; 100 µmol L⁻¹ dNTPs (Invitrogen, Carlsbad, CA, USA); 1.5 U HotStarTaq DNA polymerase (Qiagen, Valencia, CA, USA); 3 µL 10X HotStarTaq DNA polymerase PCR buffer (Qiagen). PCR conditions were: 15 min initial enzyme activation/DNA

denaturation step at 95 °C followed by 40 consecutive cycles at 94 °C for 20 s, 66 °C for 45 s and 72 °C for 15 s using an Eppendorf Mastercycler (Brinkmann Instruments Inc., Westbury, NY, USA).

Histology

The development of tubule infection after 48-h, 6- and 12-month incubation was evaluated by light microscopy. For the 48-h evaluation, two instrumented teeth were inoculated with 10⁶ cells in 15 µL THB of either *E. faecalis* OG1-S or *E. faecalis* OG1-X and incubated as previously described then split. For groups 1–6, tooth fragments were obtained from randomly selected teeth. Fragments were immersed in 10% neutral buffered formalin for 24 h and demineralized in 0.5 mol L⁻¹ ethylene diamine tetra-acetic acid (pH 7.3) paraffin-embedded, sectioned and Brown and Brenn stained. Images of sections were captured with the Olympus BX-51 microscope and DP-70 digital camera and software (Olympus, Melville, NY, USA).

Data analysis

Each tooth was scored as either positive or negative for viable *E. faecalis* under any of the growth conditions. Chi-square tests were used to compare the number of teeth positive for growth after (i) 6- and 12-month filling (groups 1 and 2), (ii) 6-month incubation with either 10⁶, 10⁵, 10⁴, or 10³ bacteria (groups 3–6) and (iii) inoculation with the gelatinase-producing *E. faecalis* OG1-S and the reduced gelatinase-producing *E. faecalis* OG1-X. Significance was set at $P < 0.05$.

Results

After 6 (group 1) and 12 months (group 2) incubation viable *E. faecalis* was recovered from all filled experimental teeth under all growth conditions ($P > 0.05$) and never from any noninoculated sterility control teeth. PCR results showed that *E. faecalis* 16S rRNA gene products were absent in all noninoculated controls and were present in all inoculated teeth. Growth of all recovered *E. faecalis* OG1-S and *E. faecalis* OG1-X was confirmed on selective media.

Initial cell count did not influence recovery ($P > 0.05$). Viable *E. faecalis* was recovered from 20, 19, 19 and 20 teeth in groups 3, 4, 5 and 6 respectively under at least one growth condition (Table 2). There was no difference in the number of

Table 2 Number of samples demonstrating positive growth of *Enterococcus faecalis* in cell density experiments

<i>Enterococcus faecalis</i> strain	Aerobic		Anaerobic		Overall group positive
	BEA	THB	THB-serum	THB-serum	
Group 3					
OG1-S	10	10	10	10	10
OG1-X	9	10	9	9	10
Group 4					
OG1-S	10	10	10	9	10
OG1-X	7	9	9	9	9
Group 5					
OG1-S	9	9	9	9	9
OG1-X	7	7	10	7	10
Group 6					
OG1-S	10	10	10	10	10
OG1-X	9	10	10	10	10
Total	71	75	77	73	78

Enterococcus faecalis OG1-S, gelatinase producing; OG1-X, reduced gelatinase producing mutant derivative of OG1-S; BEA, bile esculin agar; THB, Todd-Hewitt broth. Canals were inoculated with 10^6 cells in group 3, 10^5 cells in group 4, 10^4 cells in group 5 and 10^3 cells in group 6.

teeth with viable *E. faecalis* OG1-S compared to *E. faecalis* OG1-X ($P > 0.05$) indicating that the capacity for gelatinase production did not influence the recovery of viable *E. faecalis*.

Dentinal tubule infection was evident under light microscopy in sections from inoculated teeth after 48-h, 6- and 12-month incubation. Representative images obtained after 48-h and 12-month incubation are

shown in Fig. 1. Bacteria were seen in the dentinal tubules on the root canal side only and not from the cementum side.

Discussion

The present *in vitro* investigations showed that *E. faecalis* introduced into root canals maintained viability for 12 months without additional nutrients. Others have reported that *E. faecalis* has the capacity to survive under various environmental stresses, for example, when exposed to salt, bile salts, acid, heat (Flahaut *et al.* 1996), glucose starvation (Capiiaux *et al.* 2000), NaOCl (Laplace *et al.* 1997) and starvation in tap water (Hartke *et al.* 1998). In contrast, there is less known about the survival of *E. faecalis* over an extended period. *E. faecalis* was capable of surviving more than 90 days on commonly used hospital fabrics and plastics (Neely & Maley 2000) and for up to 4 months in water (Figdor *et al.* 2003). Other *in vitro* studies have shown that *E. faecalis* is capable of entering and recovering from the viable but nonculturable (VBNC) state, a survival strategy adopted by bacteria when exposed to environmental stress (Lleò *et al.* 2001). VBNC *E. faecalis* displayed cell wall alterations that might provide protection under unfavourable environmental conditions (Signoretto *et al.* 2000) and maintained adhesive properties to cultured human cells (Pruzzo *et al.* 2002).

Sundqvist *et al.* (1998) speculated that *E. faecalis* 'can enter the canal, survive the antibacterial treat-

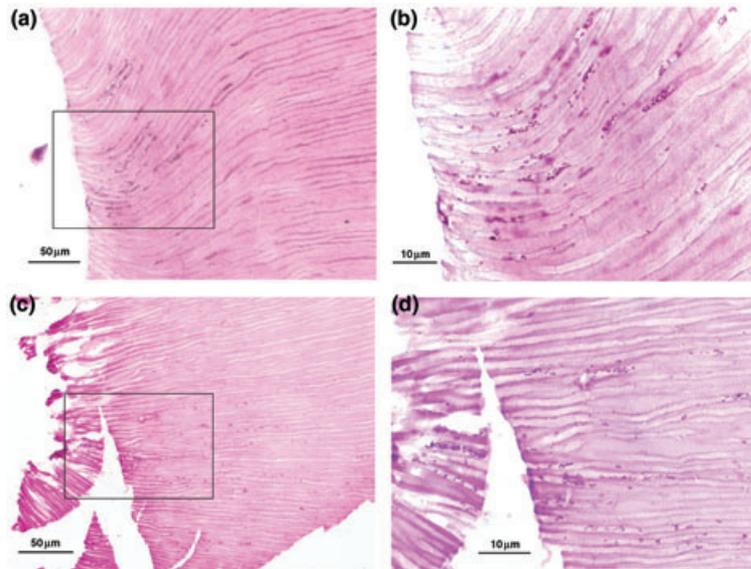


Figure 1 Infection of dentinal tubules by *Enterococcus faecalis* OG1-S after 48-h incubation (a and inset in b) and after root canal filling and 12-month incubation (c and inset in d). Brown and Brenn stain.

ment and then persist after obturation'. However, there are few data on long-term survival of *E. faecalis* in association with endodontic treatment. *E. faecalis* was able to survive in infected bovine dentine in water and in calcium hydroxide for at least 10 days (Ørstavik & Haapasalo 1990). The ability of *E. faecalis* to survive in the root canal *in vivo* is restricted to the classic monkey study by Fabricius *et al.* (1982) in which it was shown that viable *E. faecalis* was recovered from all nine (unobtured) root canals 6 months after inoculation with approximately 10^5 cells. In the present study, recovery of viable cells after 6 months was evident after an initial inoculum of 10^3 cells.

Anaerobic or aerobic conditions did not influence recovery of the facultatively anaerobic *E. faecalis* species in agreement with previous data (Figdor *et al.* 2003). In addition to oxygen conditions, other factors that did not influence recovery of *E. faecalis* were the gelatinase capacity of the *E. faecalis* strain and initial cell density. Gelatinases are extracellular zinc endopeptidases capable of hydrolyzing gelatin, collagen and other peptides that were produced by 70% of *E. faecalis* strains isolated from root canals (Sedgley *et al.* 2005b). Expression of GelE might contribute to increased dissemination of *E. faecalis* in high-density environments (Waters *et al.* 2003).

Recovery of *E. faecalis* from tooth fragments was also not influenced by the addition of human serum to growth media after splitting teeth. The addition of human serum was investigated because fluid resembling serum in the periapical tissues could potentially supply nutrients to support microorganisms left behind in or near dentine; starved *E. faecalis* cells recovered better in media supplemented with human serum (Figdor *et al.* 2003). Serum enhanced *E. faecalis* biofilm formation on gutta-percha (Takemura *et al.* 2004). In the present study, the addition of 20% human serum to THB was not essential for recovery. However, there was a high survival rate of bacteria over all conditions. Thus, whether serum enhances survival of starved *E. faecalis* would need to be tested under more challenging conditions than those in the present study, including longer incubation periods, less dense initial cell inocula, thermal obturation methods and the use of different sealers. The influence of other products potentially found in the periapical region on survival of starved *E. faecalis* could also be investigated.

In this study, bacterial suspensions were inoculated directly into the root canal rather than immersing root samples into a liquid culture and allowing growth into dentinal tubules from both root canal and cementum

sides (Haapasalo & Ørstavik 1987). It is important to note that, using the present method, bacteria could have entered lateral canals and grown and survived without nutrients on the root surface, although this was not observed histologically in 48-h, 6- and 12-month specimens. From a clinical perspective, recesses and diverticula inaccessible to biomechanical instrumentation can provide a niche for microorganisms, mostly as biofilms (Nair *et al.* 2005). The incubation period of 48 h in this study was intentionally shorter than the 2-week period used to obtain a well-infected dentine sample for studying the bactericidal action of endodontic materials (Haapasalo & Ørstavik 1987). Penetration of *E. faecalis* to a depth of 300–400 μm in bovine dentinal tubules was evident after 1 day (Haapasalo & Ørstavik 1987), similarly observed after 48 h in this study (Fig. 1). As opposed to testing the viability of large numbers of bacteria after exposure to potential antimicrobial agents, this study sought to determine if any cell would recover after a period of starvation and no attempts were made to quantify the number of surviving bacteria. *In vivo*, the number of cells required to initiate and sustain the periapical infection remains to be determined, but would be also dependent on the location of the cells and the host response.

The survival of *E. faecalis* after 'entombment' by filling reported here supports data from clinical studies (Sundqvist *et al.* 1998, Siqueira & Rôças 2004). However, with reference to the use of a zinc-oxide eugenol-based sealer (Roth), the present data contrast with those obtained using a dentine block model infected with *E. faecalis* (Saleh *et al.* 2004). Saleh *et al.* (2004) reported that Grossman's sealer (also a zinc-oxide eugenol-based sealer) killed all *E. faecalis* within 7 days in samples of circumpulpal dentine collected from the inner 300 μm of the canal; deeper samples were not obtained. In contrast, in this study, sampling was not limited to any part of the tooth. In the event of leakage alongside the root canal filling material, logically the initial source of bacteria would be closest to the root canal. It has been observed that invasion of bacteria into the root canals varies in terms of depth of penetration and not all tubules are equally invaded (Peters *et al.* 2001, Haapasalo *et al.* 2003). These observations were also borne out in light microscopy examination of infected sections that showed some dentinal tubules heavily invaded while adjacent tubules were empty (Fig. 1). The primary purpose of histological examination was to confirm the presence of bacteria in dentinal tubules at the three time periods.

There was no evidence of an increase in bacterial numbers over time. Interestingly, bacteria seemed to stain more weakly after the starvation periods. Whether these observations can be attributed to cell wall changes as a result of starvation (Signoretto *et al.* 2000) could be addressed in investigations using more sophisticated microscopic methods.

The original source of *E. faecalis* recovered from previously filled root canals undergoing retreatment is uncertain. Clinical studies have shown that *E. faecalis* usually make up a small proportion of the flora in the untreated root canal (Byström & Sundqvist 1985, Sjögren *et al.* 1997). One source of *E. faecalis* infecting the filled root canal might be the oral cavity (Rams *et al.* 1992, Sedgley *et al.* in press), potentially via coronal leakage after root canal filling. Alternatively, *E. faecalis* strains might be introduced into the root canal as contaminants during endodontic treatment procedures and bacteria left in dentinal tubules might be a reservoir from which canal reinfection can occur (Oguntebi 1994). Peters *et al.* (1995) stated that a sound obturation technique immediately after the cleaning, shaping and disinfection phases allows the remaining bacteria in the tubules to be either inactivated or prevented from repopulating the voids in the (former) canal space. They speculated that in the vast majority of cases, those bacteria appear not to jeopardize the successful outcome of root canal treatment. The present *in vitro* data indicate that *E. faecalis* has the capacity to recover from a prolonged starvation state in root filled teeth. This capacity could provide *E. faecalis* with an ecological advantage over other less hardy species that might be remaining in the filled root canal and thereby explain its recovery from intact previously treated root filled teeth (Sjögren *et al.* 1997). The clinical implications are that viable *E. faecalis* entombed at the time of root filling could provide a long-term nidus for subsequent infection if the opportunity arises.

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