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INVESTIGATION OF INFECTION AND SURVIVAL MECHANISMS HELD BY *ENTEROCOCCUS FAECALIS* (STRAIN A197A): WITH RESPECT TO ENDODONTIC DISEASE

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'That which does not kill us makes us stronger'

F. Nietzsche

INTRODUCTION AND PURPOSE

It has been well established that microorganisms are the main aetiological agents of apical periodontitis[30,79]; and the success of endodontic therapy depends primarily on elimination of the microorganisms. Endodontic therapy of teeth with or without apical periodontitis yields highly favorable results[16]. However a number of endodontically treated teeth do not heal and present with signs of apical periodontitis. *Enterococcus faecalis* is the common species cultured from such cases.

To date few studies have been conducted to describe the mechanisms employed by *E. faecalis* to predominate in endodontically treated teeth. The purpose of this thesis was to elucidate the mechanisms through which *E. faecalis* is protected from the lethal effects of a root canal medicament and sustains endodontic infection.

The test strain in this work was *E. faecalis* A197A (also named: Gel1-32 as the storage code), a root canal isolate from a persistent endodontic infection and a representative of the species[73]. The same strain has been used as a test strain in previous studies[21,52-55,63,74].

Calcium hydroxide was chosen as the test medicament as it is a medicament known to be ineffective to eliminate *E. faecalis* inside the dentinal tubules. Therefore the survival mechanisms of the bacterium would be best demonstrated using this medicament itself or simulating its alkaline effect.

Experiments in this work were mainly based on exposure of the bacterium to stressful growth conditions, adherence of the bacterium to collagen and challenge against the antimicrobial agent.

Microorganisms in the root canal system may be considered to maintain their viability in a non-hospitable environment. Besides, root canal medicaments used during endodontic therapy may create an additional stressful condition for the microorganisms. Growth in this stressful environment may directly render the microorganism to a more resistant state. Or the stressful condition may promote collagen adhesion by the microorganism. The microorganism, through adhesion to collagen, may become less susceptible to exposure to antimicrobial agents. This hypothesis is depicted in Figure 1.

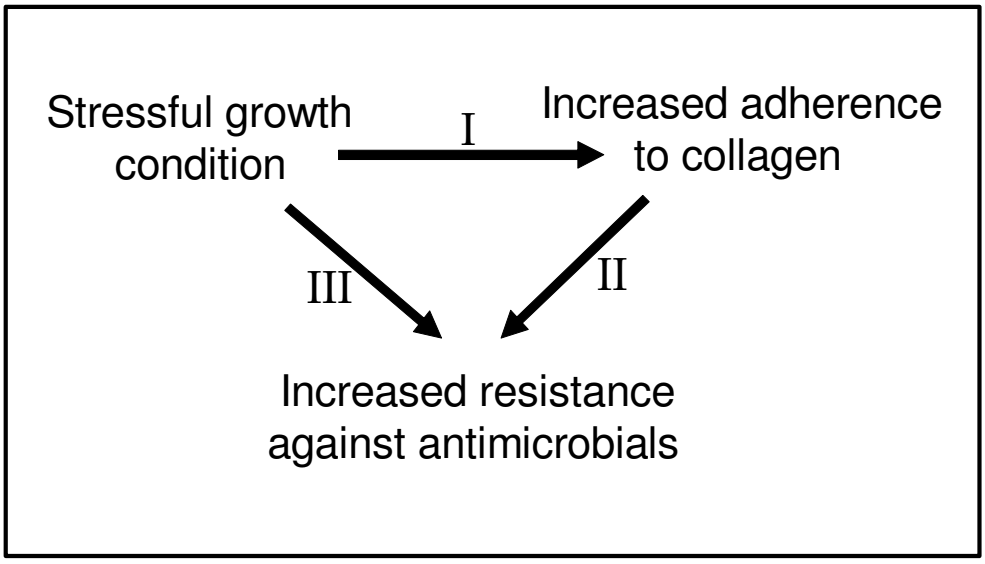

Figure 1 An overview of the hypothesis related to the infection and survival mechanisms held by *E. faecalis*

Thus, three experiments were performed to test this hypothesis. The experiments were designed to find the answers of the following questions:

Experiment I Does growth under stress increase bacterial adhesion to collagen?

Experiment II Does adhesion to collagen protect the bacterium against calcium hydroxide?

Experiment III Does growth under stress increase bacterial resistance against calcium hydroxide?

The experiments were performed at NIOM laboratories (NIOM: Scandinavian Institute of Dental Materials, Haslum, Norway)

GENERAL INFORMATION

The term 'enterococci' was first used in 1899 for Gram-positive diplococci of intestinal origin[82]. In 1906, Andrewes and Horder[2] classified some pathogenic bacteria from a patient with endocarditis in the genus *Streptococcus* as *Streptococcus faecalis*[2]. Streptococci of faecal origin were found to possess the Group D antigen according to a serological typing system developed by Lancefield[34]. In a classification scheme prepared by Sherman for the genus *Streptococcus*, *S. faecalis* was placed in the *Enterococcus* group[67]. By 1984, using nucleic acid hybridization techniques, *S. faecalis* was demonstrated to be different from the other streptococci and proposed to be transferred to a separate genus with the name *Enterococcus faecalis*[65]. To date, including *Enterococcus faecalis*, 19 species of enterococci have been identified. These are: *Enterococcus avium, Enterococcus casseliflavus, Enterococcus cecorum, Enterococcus columbae, Enterococcus dispar, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Enterococcus flavescens, Enterococcus gallinarum, Enterococcus hirae, Enterococcus malodoratus, Enterococcus mundtii, Enterococcus pseudoavium, Enterococcus raffinosus, Enterococcus saccharolyticus, Enterococcus seriolicida, Enterococcus solitarius* and *Enterococcus sulfureus*.

Enterococci inhabit the gastrointestinal tract, the oral cavity and the vagina in human as normal commensals. However, they can cause a wide variety of diseases in human, infecting the urinary tract, bloodstream, endocardium, abdomen, biliary tract, burn wounds and indwelling foreign devices[28]. Enterococci rank among the top three nosocomial bacterial pathogens[57,87], and strains resistant

to currently available antibiotics pose real therapeutic difficulties[26,35]. Up to 90% of enterococcal infections in humans are caused by *E. faecalis.* The majority of the remainder is caused by *E. faecium,* and infections with the other species are rare[28]. *Enterococcus faecalis* has also been implicated in endodontic infections. Although *E. faecalis* makes up only a small proportion of the initial flora of untreated teeth with necrotic pulps[3,60,71,80], it has been frequently found in obturated root canals exhibiting signs of chronic apical periodontitis, isolated in 30-70% of the positive cultures, and often as pure culture[19,22,48,51,81]. Molecular techniques confirm the high prevalence of *E. faecalis* in failed endodontic treatment cases, detecting the specific gene fragments of the species at a range between 60-90% of the cases[19,60,61,66,72]. Moreover, *E. faecalis* was found to be among a group of bacteria cultured from periapical lesions refractory to endodontic therapy[78].

Enterococci can withstand harsh environmental conditions. They can grow at 10°C and 45°C, at pH 9.6, in 6.5% NaCl broth and survive at 60°C for 30 minutes[67]. *E. faecalis* can adapt to adverse conditions: following pre-exposure to sub-lethal conditions, *E. faecalis* becomes less sensitive to normally lethal levels of sodium dodecyl sulphate, bile salts, hyperosmolarity, heat, ethanol, hydrogen peroxide, acidity and alkalinity; furthermore, 'cross protection' is pronounced against diverse challenges[12-15]. Starving cells of *E. faecalis* maintain their viability for extended periods and become resistant to UV irradiation, heat, sodium hypochlorite, hydrogen peroxide, ethanol and acid[17,23]. *E. faecalis*, moreover, can enter the viable but non-cultivable (VBNC) state, a survival

mechanism adopted by a group of bacteria when exposed to environmental stress, and resuscitate upon returning to favourable conditions[37].

E. faecalis has been shown to invade dentinal tubules in *in vitro* studies[1,20,39,46,50,85] however not all bacteria have this ability[1,49]. In animal studies where pure cultures of various bacteria were inoculated separately into root canals, *E. faecalis*, unlike others, was found to colonize the root canal in most cases and survive without the support of other bacteria[10,75]. *E. faecalis* is resistant against the antimicrobial effects of calcium hydroxide, a strongly alkaline disinfectant[4,7,20,46,70,74,76,85], probably partly due to an effective proton pump mechanism which maintains optimal cytoplasmic pH levels[9] and also due to cell membrane durability[41]. Root canal sealers containing calcium hydroxide were also found to be ineffective against *E. faecalis*[32]. Furthermore, *E. faecalis*, intrinsically or via acquisition, may be resistant to a wide range of antibiotics[26,35].

E. faecalis possesses a group of virulence factors which make it a potent microorganism to cause diseases. These virulence factors are: aggregation substance, surface adhesins, sex pheromones, extracellular superoxide, the lytic enzymes gelatinase and hyaluronidase, and the toxin cytolysin. In addition, not strictly considered virulence factors, AS-48 and a number of bacteriocins facilitate the predominance of *E. faecalis* in a mixed infection exerting lytic activity on a broad range of Gram-positive and Gram-negative bacteria. These factors have been linked to the establishment and sustenance of endodontic infections; and they have been discussed elsewhere[31].

The root canal space is considered hardly a nutrient-rich medium for bacterial growth. However *E. faecalis* in the non-vital root canal can feed on host factors such as serum[11,39] which may originate from the alveolar bone and the periodontal ligament and leak into the root canal through the apical foramen. Furthermore, hyaluronidase as one of the virulence factors of *E. faecalis* may act to supply nutrients for the bacterium[27]. The target substrate for hyaluronidase is hyaluronic acid (hyaluronate, hyaluronan); and this is present in dentine[5,29]. Disaccharides as the degradation products of hyaluronic acid may constitute the source of energy for bacterial growth. And also, with its high content of organic substances, smear layer produced on instrumented root canal surfaces[83] may be a substrate for bacterial growth.

Adhesion and colonization of the host by microorganisms is the first step in the establishment of most infectious diseases, after which pathological changes occur. Adherence of *E. faecalis* to extracellular matrix proteins, including collagen type I, has been demonstrated[39,42,43,56,62,88,89]. Adhesion to collagen type I by bacteria is of particular importance since this is the main organic component of dentine[36]. Increased adhesion of various clinical strains of *E. faecalis* to extracellular matrix proteins was termed 'conditional adherence'; because these strains adhered significantly more after they were grown at 46°C- a representative stressful condition for bacterial growth[88]. It was found that a special adhesin for collagen, called 'Ace', was produced by *E. faecalis* when it was grown specifically at 46°C[42,43,56]. Our pilot tests, using a clinical isolate of *E. faecalis* (strain A197A: possesses the gene for the production of Ace[55]), revealed similar

results to these findings in terms of conditional adherence to collagen type I. Therefore, we decided to examine the effects on the adherence of *E. faecalis* of other stressful conditions which can take place in the medicated root canals. As calcium hydroxide (a highly alkaline medicament) fails to eliminate *E. faecalis* in the root canal, the alkalinizing effect of this medicament could have some effect on the adhesiveness of the bacterium. Thus, the purpose of the experiment I was to evaluate the effect of growth at pH levels from 7.1 to 9.5 on the adherence of *E. faecalis* to organic surfaces, namely collagen type I and bovine serum albumin, and to compare the results with those obtained for the 46°C-grown bacteria.

Calcium hydroxide is a common medicament used in endodontic therapy. With its high pH of 12.5 it is a strong antimicrobial. Although calcium hydroxide effectively kills endodontic pathogens, including *E. faecalis*, in experimental conditions where dentine is not included[8], it fails to eliminate *E. faecalis* in those where dentine is present[20,21,46,52,70,74,85]. The collagen adherence phenomenon for *E. faecalis* raised the question if this might confer resistance to the bacterium against root canal medicaments. Such resistance (if exists) might help explain why calcium hydroxide was ineffective in killing *E. faecalis* in dentinal tubules or in experimental conditions where dentine was present. Therefore the purpose of the experiment II was to determine whether cells of *E. faecalis* adhering to collagen type I were more resistant to challenge with calcium hydroxide.

As mentioned earlier in the text, cells of *E. faecalis* were found to become less susceptible to various challenges following brief exposure to a milder

dose; and cross protection, as well as homologous tolerance, was pronounced against diverse challenges[12-15]. Similar adaptation may be expected for bacteria growing at a stressful condition. The purpose of the experiment III was to compare the resistance against calcium hydroxide of cells of *E. faecalis* grown at a stressful condition with those grown at an optimum condition.

MATERIALS AND METHODS

EXPERIMENT I

Bacterial strain and culture conditions

The bacterial strain used in this experiment was *E. faecalis* A197A, a clinical isolate from a persistent endodontic infection[73]. Tryptone Soya Broth (TSB, Oxoid Ltd, Basingstoke, England) was used as the basic growth medium. Media of pH 7.5, 8.0, 8.5, 9.0 and 9.5 were prepared by the addition of 1 M NaOH to TSB. Colonies were picked from solid media (TSB agar) and inoculated into each medium. Incubation was at 37°C overnight; one inoculated part of the pH 7.1 medium was incubated at 46°C. The cultures were harvested by centrifugation at 10.000 g for 10 min at 4°C, washed 3 times with equal amounts of phosphate-buffered saline (PBS, pH 7.4) and finally resuspended in PBS. The purity of the bacterial cultures was routinely checked during the experiments by Gram staining and observing the colony morphology.

Growth curves pertaining to each growth condition were determined through measurement of broth turbidity with a spectrophotometer (Multiskan EX, Labsystems, Finland) at a filter wavelength of 540 nm.

Adherence assay

Three hundred μL of bovine serum albumin (100 μg/mL PBS) (Sigma Chemical Co, St Louis, USA) or collagen type I (50 μg/mL PBS) (Sigma)

was added to wells of 96-well microtiter plates (Sarstedt Inc, Newton, USA) and incubated at 4°C overnight. The wells were decanted, rinsed 3 times with sterile PBS, blocked with 300 µL of BSA (100 µg/mL PBS) at 4°C for 1 h, and rinsed again.

Bacterial adhesion to the coated surfaces was quantified employing an assay described elsewhere[47], however with some modifications. Briefly, 200 µL of bacterial suspensions of an optical density OD_{540}=1.0 (approximately $2.0x10^9$ bacteria/mL) was added to each well and incubated at 37°C for 2 h. Wells coated with BSA or collagen, and not filled with the bacterial suspensions served as controls. The wells were rinsed 3 times with PBS to remove unbound bacteria and the plate was dried in an inverted position. The wells were stained with 325 µL of 1% crystal violet for 20 min, rinsed with PBS and then dried again. Bound crystal violet was solubilized by addition of 200 µL of ethanol-acetone (4:1, vol/vol) and the optical density at 570 nm of the dissolved dye was measured. Each assay was performed in four parallels and repeated twice.

The relationship of the OD measurements of the dissolved crystal violet to bacterial numbers was assessed in a separate methodological experiment: suspensions of known bacterial concentrations were dried onto the sides of wells prior to the staining procedure. The ensuing OD readings were used to construct standard curves for OD *vs* bacterial cell concentrations. A near-linear relationship was found for a log-log plot of the results (Figs. 2 and 3), which was used to transform the OD measurements to bacterial cell concentrations. The data obtained were analyzed using Kolmogorov-Smirnov test for normality, Levene's

test for equality of variances and Student's *t*-test, with α=0,05 as the level for statistical significance.

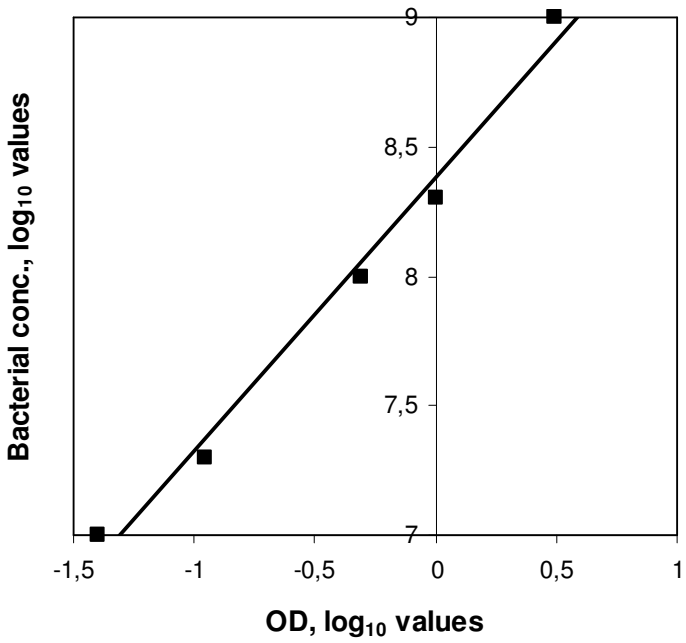

Figure 2 The standard curve constructed by intersecting known concentration of bacteria and OD readings at 570 nm (for collagen-coated wells)

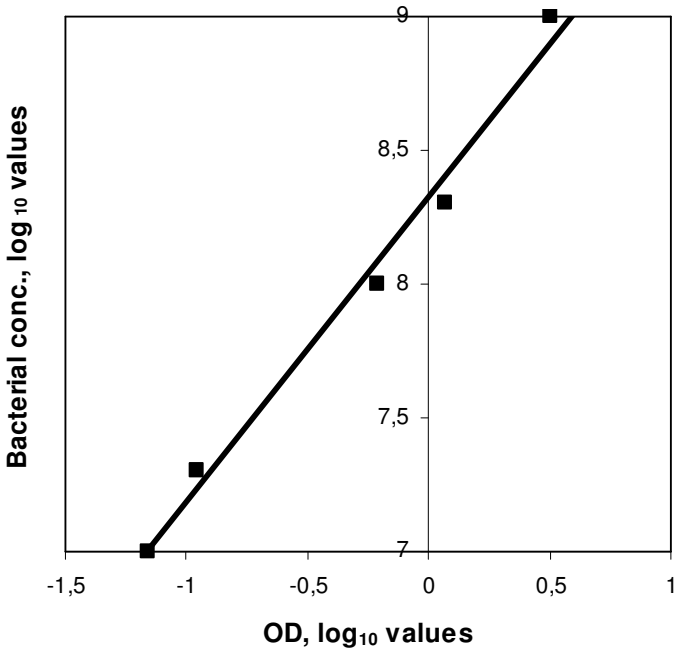

Figure 3 The standard curve constructed by intersecting known concentration of bacteria and OD readings at 570 nm (for BSA-coated wells)

Scanning electron microscopy

A separate plate was not stained with CV but was used for SEM examinations. The wells were kept in 10% neutral buffered formaldehyde solution overnight, rinsed thoroughly with distilled water and dried at 50°C for an hour. The bottoms of the wells were separated using a diamond disc at low speed. The specimens were mounted on aluminium stubs with conducting carbon cement, sputter coated with gold-palladium and observed in a scanning electron

microscope (Phillips XL 30 ESEM, Eindhoven, The Netherlands). The pattern of adherence in terms of the presence of chains and clusters of bacteria, as well as area coverage, was examined.

EXPERIMENT II

Bacterial strain and culture conditions

The bacterial strain used in this experiment was *E. faecalis* A197A, a clinical isolate from a persistent endodontic infection[73]. Tryptone soya broth (TSB, Oxoid Ltd, Basingstoke, England) was used as the growth medium. Glycerol stock of the bacteria was prepared by growing cells overnight in TSB at 37°C. The cells were harvested by centrifugation (10.000 g, 10 min, 4°C) and resuspended in fresh TSB and 80% glycerol (1:1). This stock suspension was stored at -20 °C until required.

A 2% inoculum from the glycerol stock was grown in fresh TSB at 46°C to the early stationary phase. The cells were centrifuged 3 times (10.000 g, 10 min, 4°C), washed with phosphate-buffered saline (PBS, pH 7.4) and finally resuspended in PBS. The bacterial density was adjusted to OD_{540}=1.0 using a spectrophotometer (Multiskan EX, Labsystems, Finland). Aliquots (1050 μL) of the bacterial suspension were dispensed to plastic test tubes. There were three test groups.

Experimental design

Group 1 was pre-treated with collagen and challenged with pure calcium hydroxide. Group 2 was pre-treated with PBS and collagen was added during challenge with calcium hydroxide. Group 3 was pre-treated with PBS and exposed to pure calcium hydroxide (no addition of collagen).

Pre-treatment conditions

Pre-treatment was at 37°C for 1 h. Group 1 was pre-treated with 1050 µL of collagen type I in PBS (100µg/mL) (Sigma, USA). The pH of this solution was 4.25. Groups 2 and 3 were pre-treated with 1050 µL of PBS, the pH of which was adjusted to 4.25 by addition of acetic acid (Fig.4).

After 1 h, 100 µL was taken from each group, serially diluted, seeded on TSA plates and incubated at 37°C for 48 h. Colony forming units were counted and recorded as the initial viable bacterial count.

Challenge conditions

The test groups were challenged with saturated solution of calcium hydroxide (Merck, Darmstadt, Germany). Group 2 was challenged with 1.25 mL of saturated solution of calcium hydroxide and 5.75 mL of distilled water (solution of 7 mL). To determine whether the protective effect of collagen (if exists) stems from the adherence phenomenon or simply from the presence of collagen in the medium, 1 mL of collagen (100 µg/mL PBS) was added to Group 2 in this step. Groups 1 and 3, similar to Group 2, were challenged with 1.25 mL of saturated solution of calcium hydroxide and 5.75 mL of distilled water (solution of 7 mL). However, to compensate for the acidification of the test medium in Group 2 (because collagen was added), 1 mL of PBS adjusted to pH 4.25 was added to Groups 1 and 3 (Fig.5).

The tubes were incubated at 37°C. One hundred μL were pipetted from each tube at 1h, 6h, 12h and 24 h periods, serially diluted and seeded on TSA plates. The number of colony forming units was counted following incubation at 37°C after 48 hours. The experiment was repeated twice. Using $\alpha=0.01$ as the level for statistical significance, the data obtained were analyzed using Student's *t*-test.

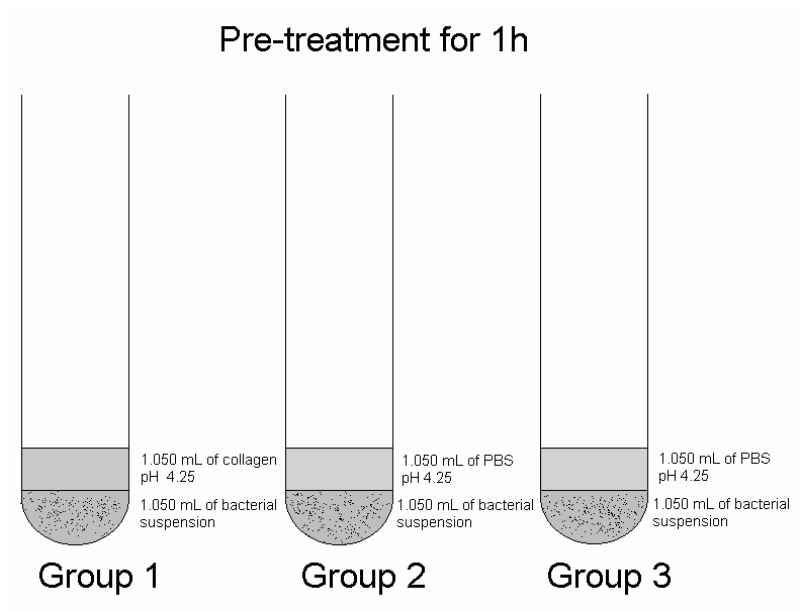

Figure 4 Presentation of the experimental design (Exp.II) for the pre-treatment step

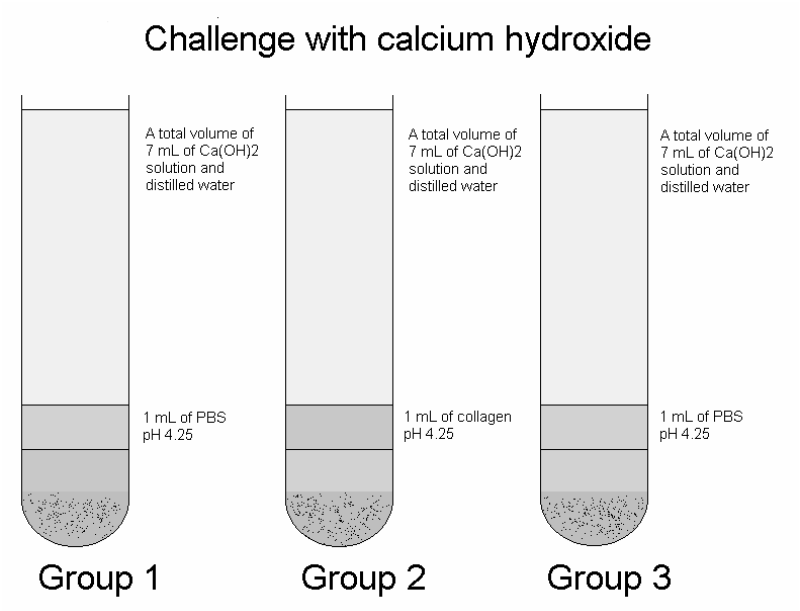

Figure 5 Presentation of the experimental design (Exp.II) for the challenge step

EXPERIMENT III

Bacterial strain and culture conditions

The bacterial strain used in this experiment was *E. faecalis* A197A, a clinical isolate from a persistent endodontic infection[73]. Tryptone soya broth (TSB, Oxoid Ltd, Basingstoke, England) was used as the growth medium. Glycerol stock of the bacteria was prepared by growing cells overnight in TSB at 37°C. The cells were harvested by centrifugation (10.000 g, 10 min, 4°C) and resuspended in fresh TSB and 80% glycerol (1:1). This stock suspension was stored at -20 °C until required.

A 2% inoculum from the glycerol stock was grown in fresh TSB at 37°C or 46°C to the early stationary phase. The cells were centrifuged 3 times (10.000 g, 10 min, 4°C), washed with phosphate-buffered saline (PBS, pH 7.4) and finally resuspended in PBS. The bacterial density was adjusted spectrophotometrically (Multiskan EX, Labsystems, Finland).

Challenge conditions

One mL aliquots of the bacterial suspension were dispensed to plastic test tubes. Saturated solution of calcium hydroxide (Merck, Darmstadt, Germany) was diluted with distilled water (1:6) and 1 mL of this was added to the test tubes.

The tubes were incubated at 37°C. One hundred µL were pipetted from each tube at 1h, 6h, 12h, 18h and 24 h periods, serially diluted and seeded on

TSA plates. The number of colony forming units was counted following incubation at 37°C after 48 hours. The experiment was repeated twice. The data obtained were analyzed using Mann Whitney U and Wilcoxon tests. Statistical significance was set at 0.01.

RESULTS

EXPERIMENT I

Growth curves pertaining to each group are shown in Figs 6 to 12. Bacterial yields suggested that growth at pH 8.5, 9.0 and 9.5 and at 46°C (but not at 7.5 and 8.0) was stressful, as significantly less turbidity occurred at these conditions compared to pH 7.1 - 37°C-grown cultures ($p<0.05$).

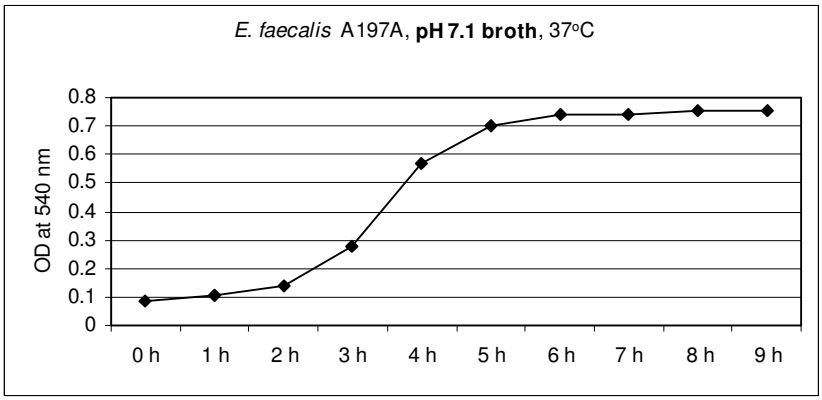

Figure 6 Growth curve for the pH 7.1 group at 37°C

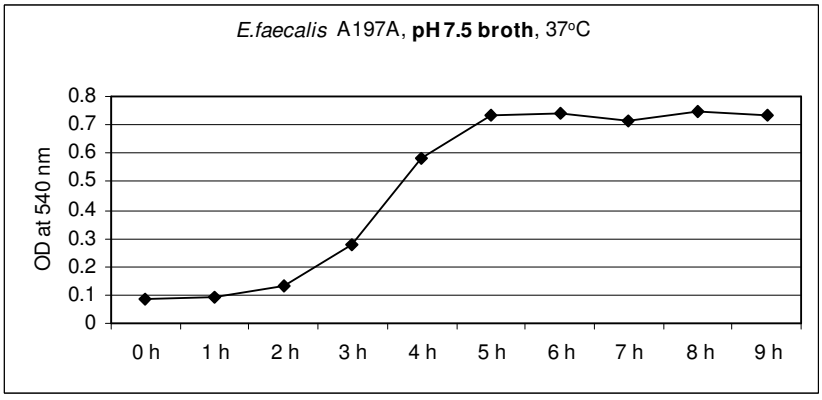

Figure 7 Growth curve for the pH 7.5 group at 37°C

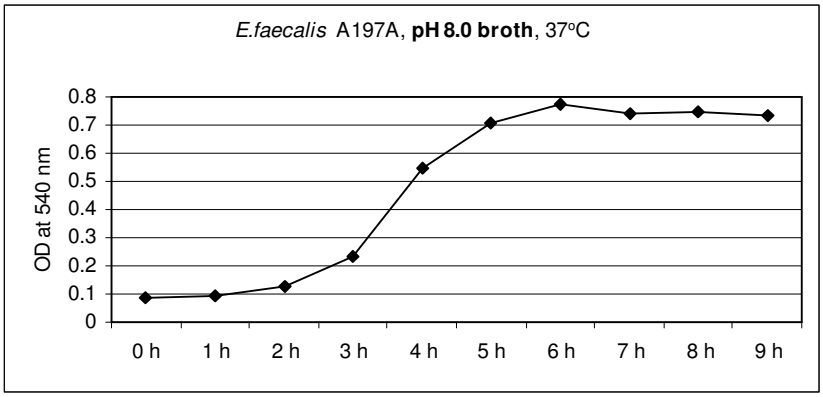

Figure 8 Growth curve for the pH 8.0 group at 37°C

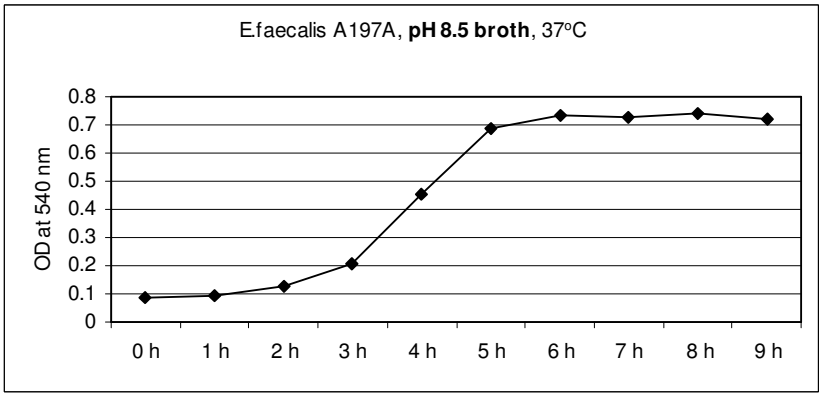

Figure 9 Growth curve for the pH 8.5 group at 37°C

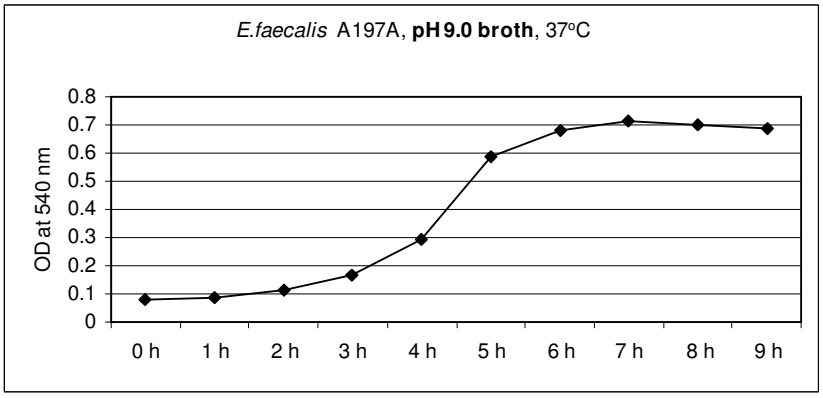

Figure 10 Growth curve for the pH 9.0 group at 37°C

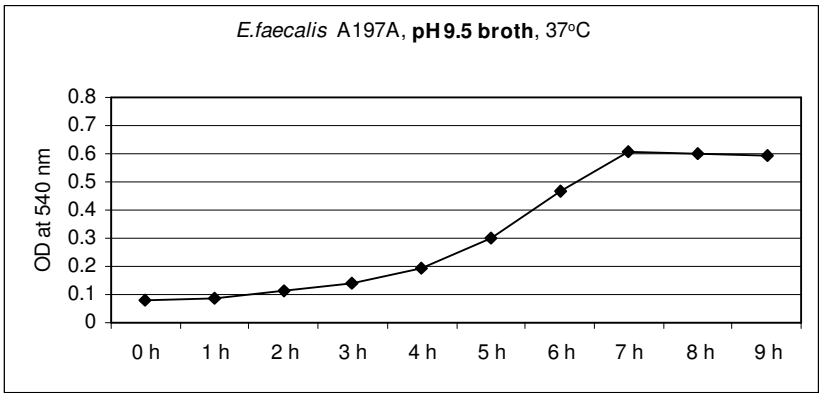

Figure 11 Growth curve for the pH 9.5 group at 37°C

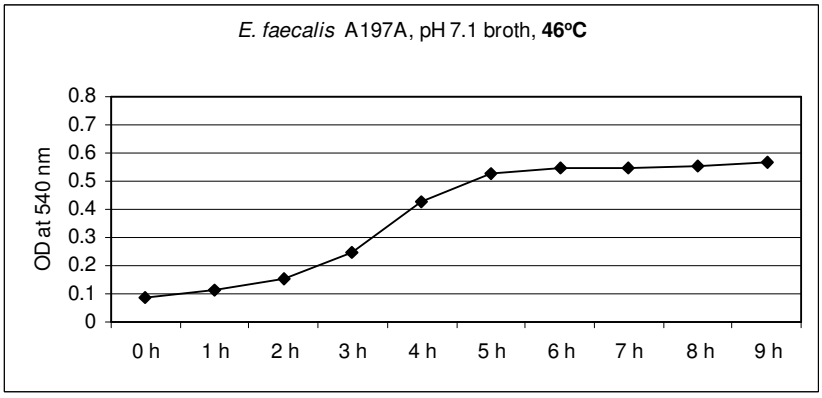

Figure 12 Growth curve for the pH 7.1 group at 46°C

No difference was found between the background staining of BSA and collagen as determined by the comparison of BSA and collagen-coated wells free of bacteria.

The experimental results are summarized in Figs.13 and 14.

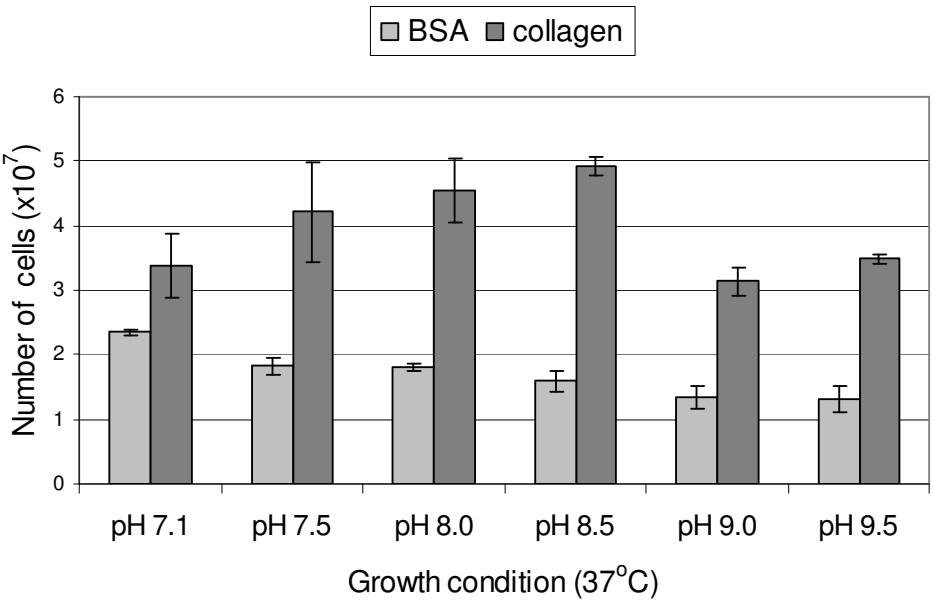

Figure 13 Number of bacteria adhering to BSA- or collagen-coated surfaces. Bacteria grown at different pHs

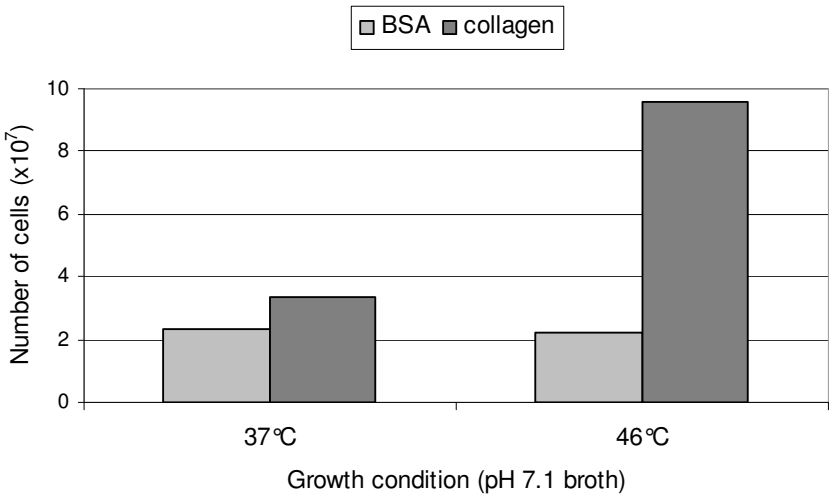

Figure 14 Number of bacteria adhering to BSA- or collagen-coated wells. Bacteria grown at different temperatures

Adherence to BSA-coated surfaces

Bacterial adherence to BSA-coated surfaces decreased steadily from pH 7.1 group down to pH 9.5 group. The pH 7.1-grown bacteria bound to BSA significantly more than the other pH groups.

Adhesion to BSA-coated surfaces for bacteria grown at 37°C or 46°C was not significantly different from each other.

Adherence to collagen-coated surfaces

Bacterial adherence to collagen-coated surfaces increased gradually from pH 7.1 group up to pH 8.5 group, which had the maximum number of binding bacteria observed among the different pH broth groups. Bacteria grown at pH 8.0 and pH 8.5 bound to collagen significantly more than those grown at pH 7.1, pH 9.0 and pH 9.5. Adherence to collagen of bacteria grown at pH 9.0 and pH 9.5 were not significantly different from the adherence of bacteria grown at pH 7.1.

Bacteria grown at 46°C bound to the collagen-coated surfaces significantly more than the 37°C-grown bacteria and any pH group.

Difference between the number of bacteria binding to BSA and collagen separately for each pH group and also for each temperature group was also statistically analyzed. Except for the pH 7.1 group grown at 37°C, the number of bacteria binding to collagen was significantly higher than the number of bacteria binding to BSA in all groups.

Scanning electron microscopy

Presence of bacteria was evident for all specimens examined except the BSA- and collagen-coated control wells. The bacteria were observed as single cells, pairs, chains or clusters (Fig.15-24). While bacteria adhering to collagen appeared to occur in clusters of varying size evenly spread out over the surface (Fig. 15,16,20-22) bacteria adhering to BSA surfaces were more irregularly distributed and occupied limited areas, with bands or strands of cells criss-crossing the surface (Fig.17-19,23,24).

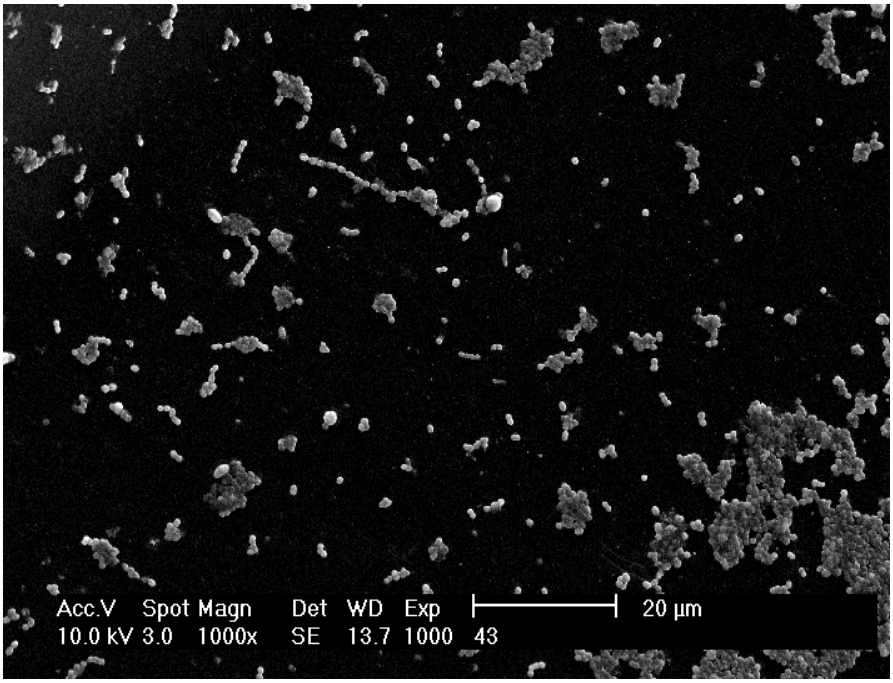

Figure 15 Adhesion of bacteria grown at pH 7.1 to the collagen-coated surface

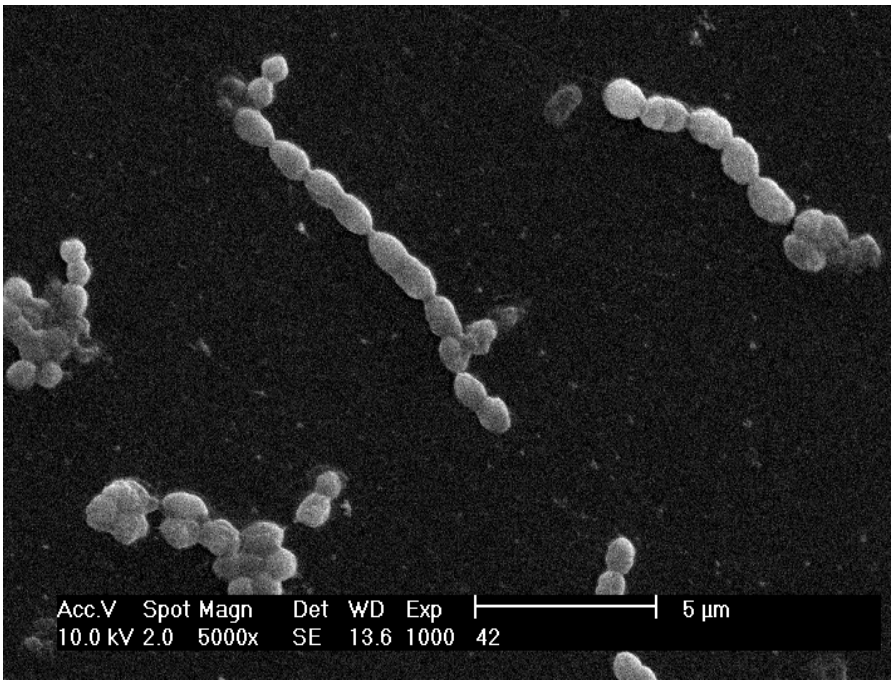

Figure 16 Adhesion of bacteria grown at pH 7.1 to the collagen-coated surface

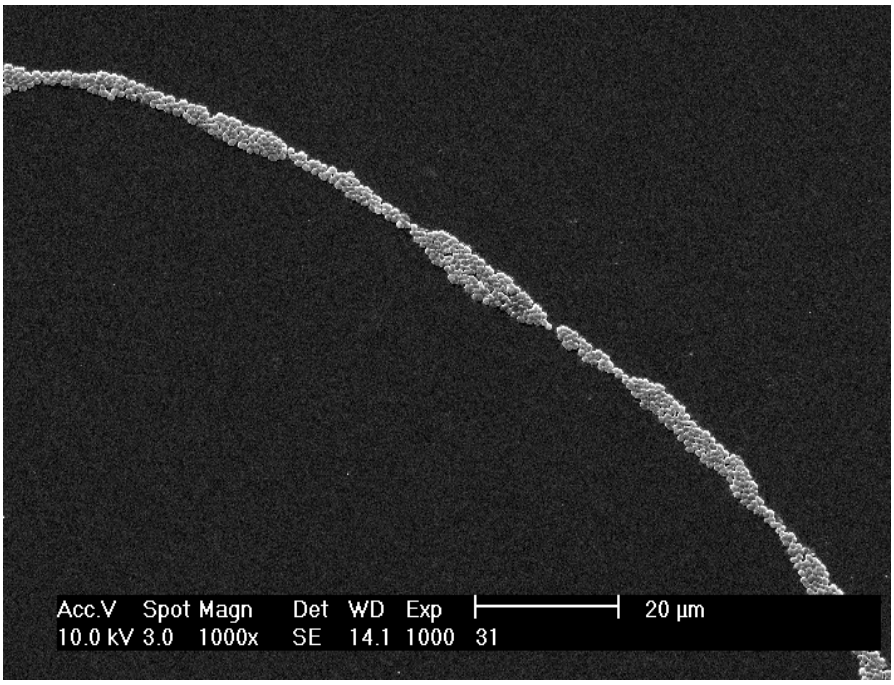

Figure 17 Adhesion of bacteria grown at pH 7.1 to the BSA-coated surface

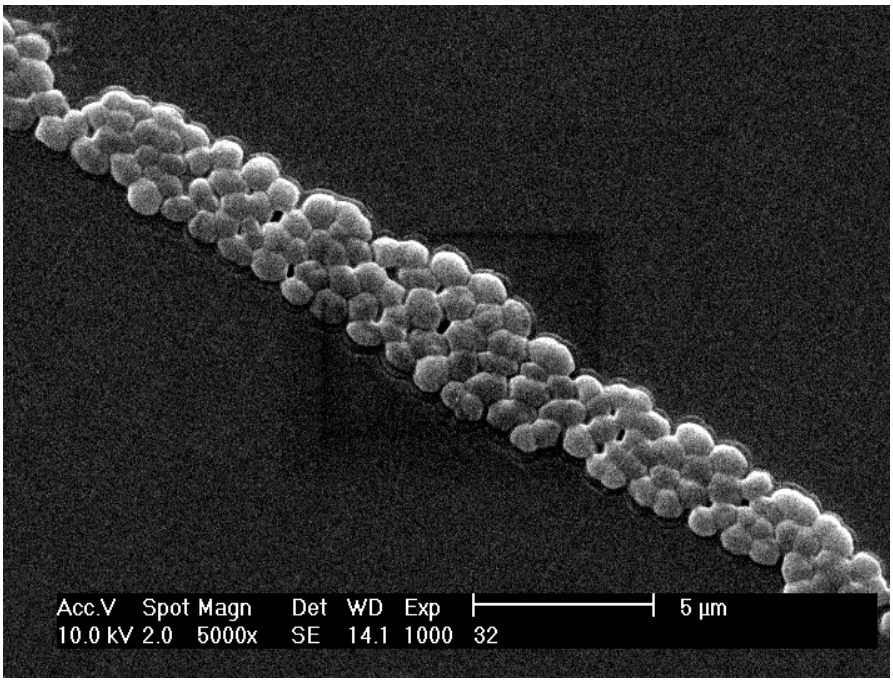

Figure 18 Adhesion of bacteria grown at pH 7.1 to the BSA-coated surface

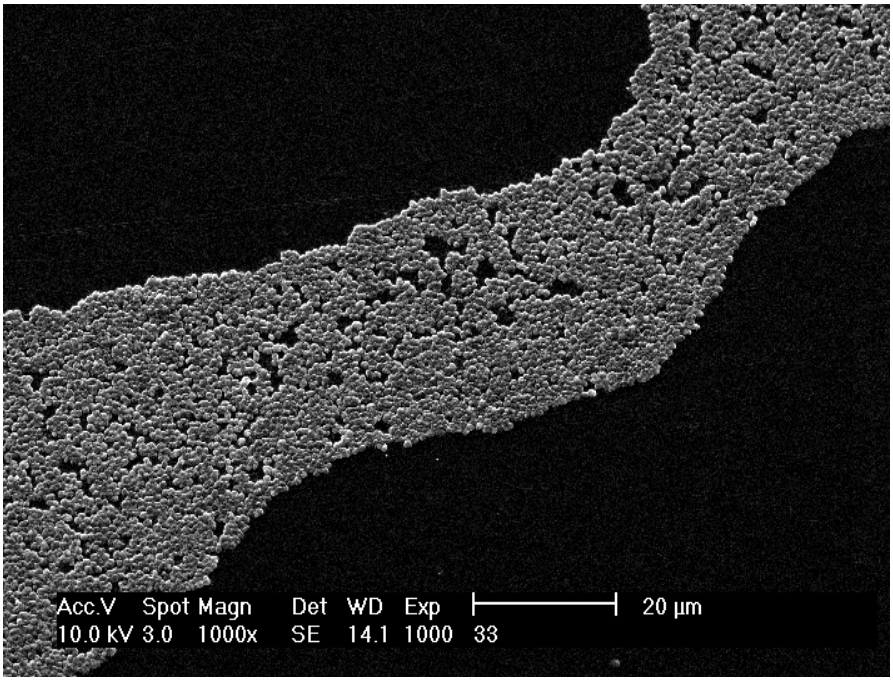

Figure 19 Adhesion of bacteria grown at pH 7.1 to the BSA-coated surface

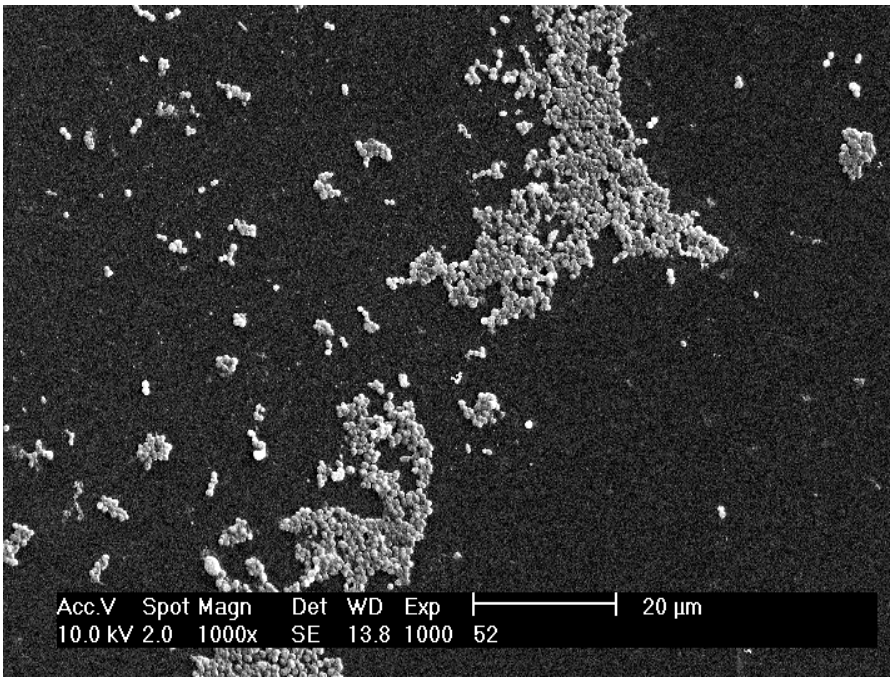

Figure 20 Adhesion of bacteria grown at pH 8.5 to the collagen-coated surface

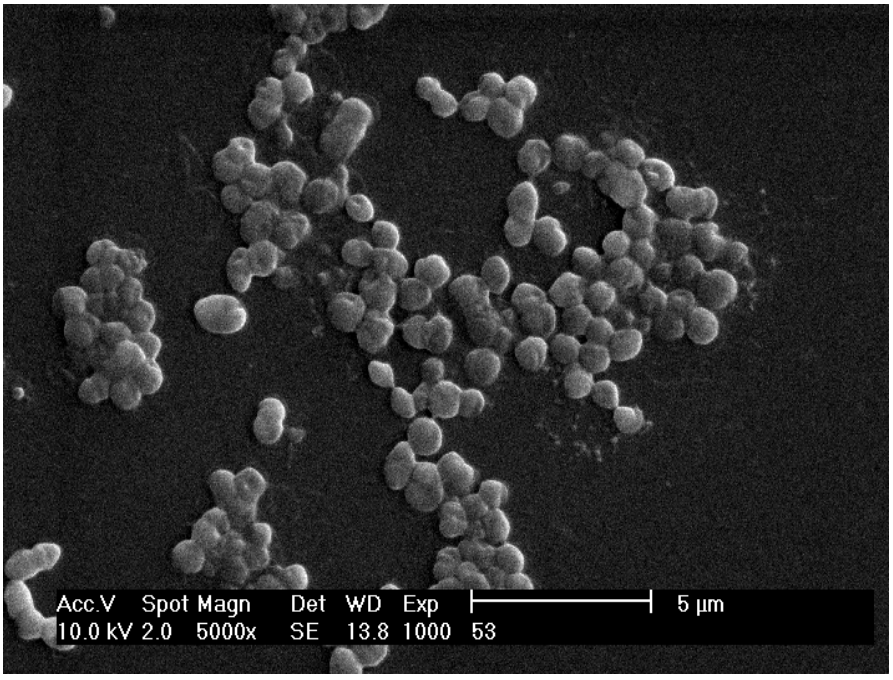

Figure 21 Adhesion of bacteria grown at pH 8.5 to the collagen-coated surface

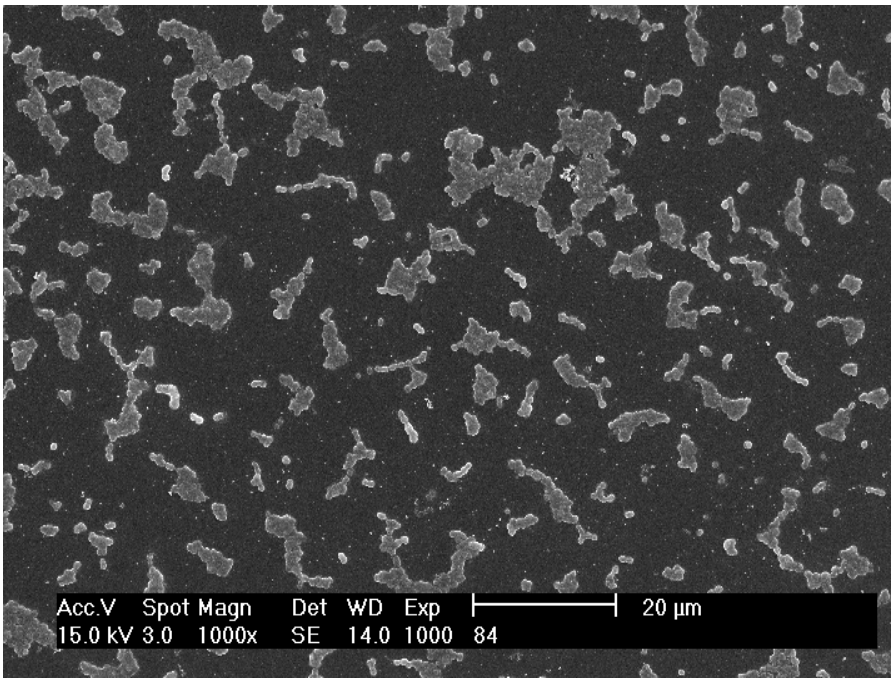

Figure 22 Adhesion of bacteria grown at 46°C to the collagen-coated surface

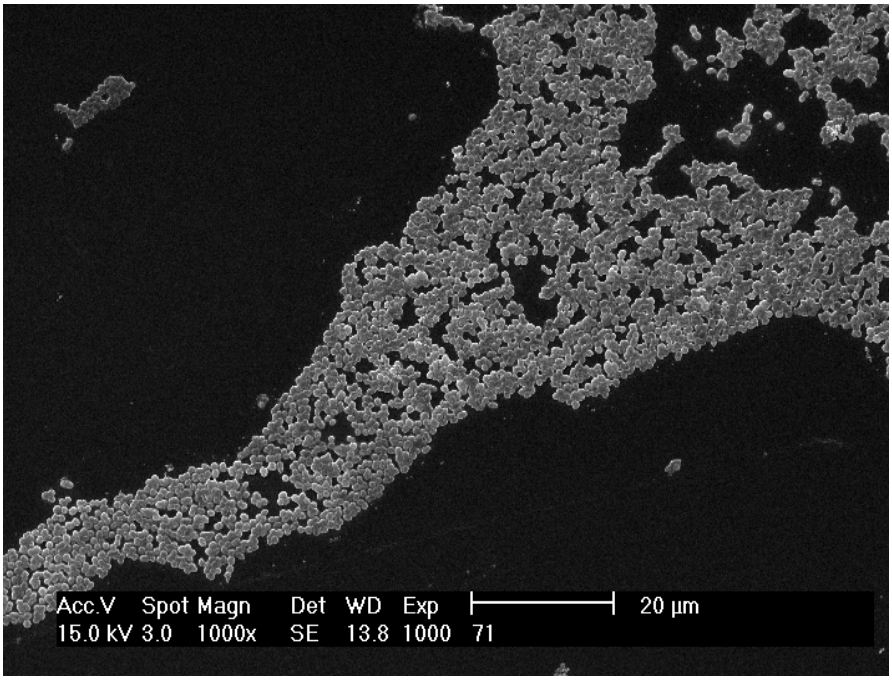

Figure 23 Adhesion of bacteria grown at 46°C to the BSA-coated surface

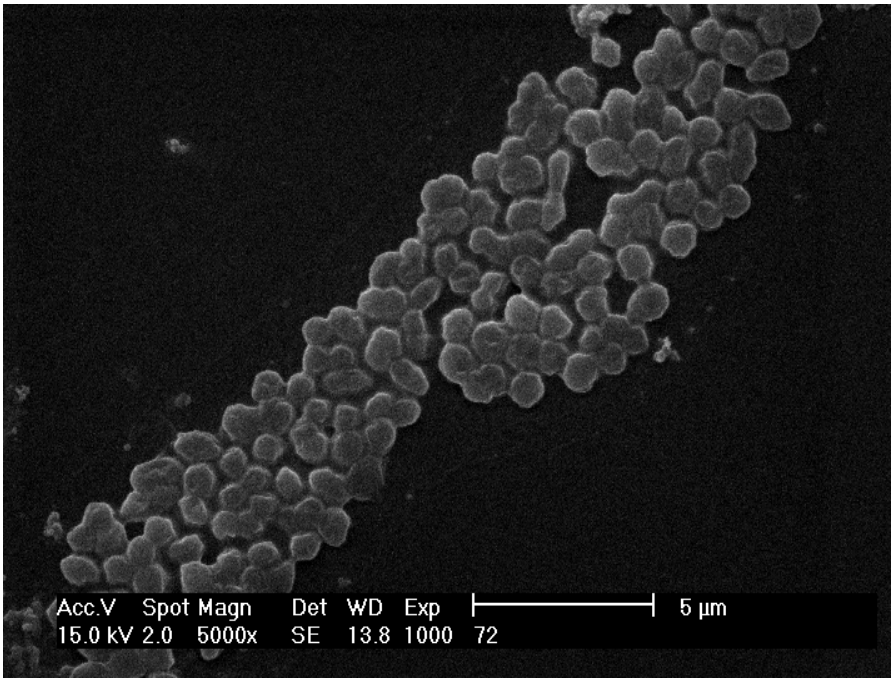

Figure 24 Adhesion of bacteria grown at 46°C to the BSA-coated surface

EXPERIMENT II

The results are shown in Fig.25. No significant difference was found for the initial counts of viable bacteria between the test groups following pre-treatment. There was no significant reduction in bacterial viability even at 6 h, and the number of viable bacteria was not significantly different among the groups at this period. However, beginning at 12 h the number of viable bacteria decreased significantly; more viable bacteria were recovered after 12 h in Group 1 compared with Groups 2 and 3. This difference was statistically significant. There was no significant difference between Group 2 and Group 3. No bacteria could be cultivated in any group at 24 h.

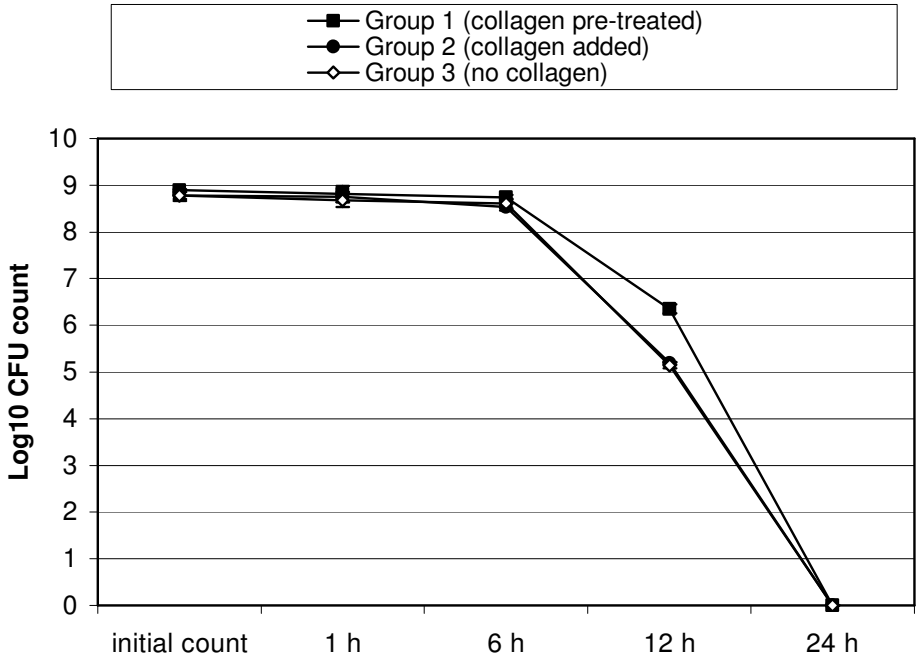

Figure 25 Survival of bacteria pre-treated with collagen or not

EXPERIMENT III

The results are shown in Fig. 26. The number of viable bacteria remained little affected ($p>0.01$) as long as 6 h for the 46°C-grown group, and as long as 1 h for the 37°C-grown group. There was a statistically significant difference between the two groups at 6 h and 12 h, and a definite difference at 18 h where no viable bacteria were cultivated in the 37°C-grown group. No viable bacteria could be cultivated in any group at 24 h.

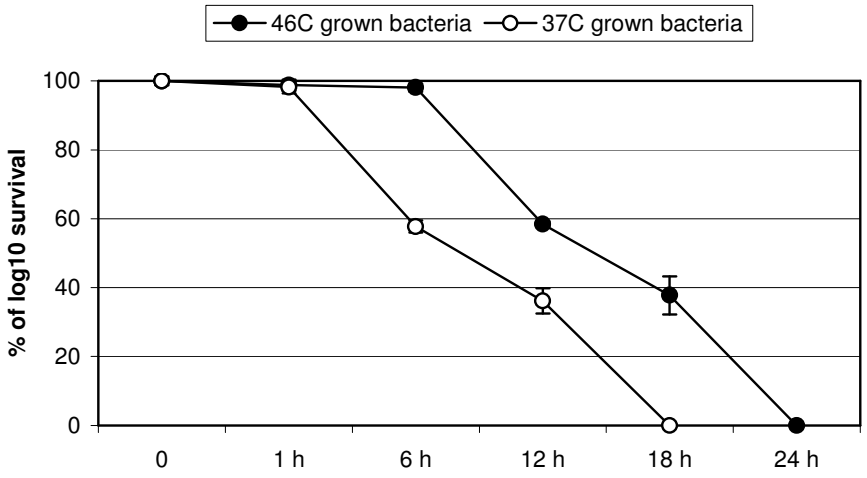

Figure 26 Survival of 46°C-grown and 37°C-grown bacteria following calcium hydroxide challenge

DISCUSSION

The major findings in experiment I were that the adhesion of *E. faecalis* A197A to BSA-coated surfaces decreased inversely with an increase in alkalinity of the growth medium and that, contrarily, the adhesion to collagen-coated surfaces increased parallel to an increase in alkalinity of the growth medium with a peak at pH 8.5, which was determined to be a stressful growth condition. Furthermore, a high-level collagen binding was observed for the bacteria grown at 46°C, which was also shown to be a stressful growth condition.

E. faecalis has been shown to invade the dentinal tubules in several studies[1,20,39,46,50,85] and this has been related to its ability to adhere to collagen[39]. It has been reported that adhesins on the cell surface, such as 'aggregation substance' and 'Ace' mediate adhesion of *E. faecalis* to collagen[56,62]. The strain (A197A) tested in this study is known to possess the gene for the production of the adhesin, Ace[55]. In the 46°C-grown group, the observed high-level binding to the collagen-coated surfaces was consistent with the term, so called: conditional adherence[88], and the adhesin that mediated the binding was most probably the Ace (produced specifically during growth at 46°C). The importance of Ace as an adhesin that aids *E. faecalis* also to bind to dentine was shown in previous studies[25,33]. While the aggregation substance and Ace are the well-characterized adhesins of *E. faecalis*, approximately 200 different proteins have been detected following exposure of *E. faecalis* to various stressful conditions, including alkaline stress[15]. The function of most of these proteins, however, is still unknown[18,58]; and perhaps some of them may be adhesins.

Besides adhesins, physicochemical characteristics of the bacterial cell surface such as surface tension, surface charge and hydrophobicity may act in the context of bacterial adhesion to solid surfaces. In one study, the collagen adhesion of *E. faecalis* was linked to its cell surface hydrophobicity[89]. Change in the environmental pH as a stimulus has been known to alter the physicochemical characteristics of bacteria as well as to trigger expression of various genes associated with virulence or metabolic regulations[45]. Thus, in addition to the possibility of synthesis of adhesins by the bacterium, it is also possible that change in the bioenergetic parameters of the cell surface might have increased its adherence to the collagen-coated surfaces. However, it remained unknown in this study by which means the pH 8.0- and pH 8.5-grown bacteria exhibited increased adherence to the collagen surfaces.

Calcium hydroxide, with its high pH of 12.5, is a strong antimicrobial substance, especially when it is in contact with microorganisms. However, when dentine is included in test systems, its effectiveness is decreased[20,46,70,74,85]. It has been shown that dentine powder inhibits the antimicrobial effect of calcium hydroxide against *E. faecalis*[21,52]. Also, there can be variations in alkalinizing potential of various formulations of calcium hydroxide[64]. Moreover, there are problems in efficient delivery of calcium hydroxide into root canals, especially to the apical third[6,59,69]. Therefore, it seems likely that sufficient levels of alkalinity cannot be achieved in all parts of the root canal and probably only a slight increase in pH may occur. Thus, the pH rise may not be sufficient to kill, but may actually promote the collagen mediated adhesion

of the bacteria which in turn leads to dentinal tubule invasion. Effects of mild alkalinity may be pronounced also for the bacteria already established within the dentinal tubules. The pH distribution through dentine has been reported to vary, being higher in the inner dentine and gradually decreasing towards the outer regions, and being lower in the apical dentine than in the cervical region[44,84]. As a result, bacteria within certain depths of the dentine may be exposed only to mild levels of alkalinity. As binding to collagen would increase the dentinal tubule invasion and therefore the depth of infection by *E. faecalis*, the invading bacteria will become less sensitive to further disinfection procedures since they will be protected in deeper locations.

In pulpless teeth, there may be ingress of tissue fluids through the apex from periradicular tissues. Albumin is a major protein component of tissue fluids. *E. faecalis* has previously been found to bind to albumin with the involvement of protein and/or carbohydrate containing components as the surface receptor[68]. In the study presented here, after growth at neutral pH, no difference was observed between the adherence of the bacteria to albumin or collagen-coated surfaces. However this changed in favour of adherence to collagen as the pH of the growth medium increased, suggesting that the bacteria would preferentially bind to the collagen moiety of the dentine instead of being blocked by the albumin component of the serum. The most striking difference between binding to collagen or BSA was after growth at pH 8.5, but the difference was still significant after growth at lower and higher pH values. In one study, the presence of albumin in the growth medium of *E. faecalis* JH2-2, however, did not impede

in the ability of the bacteria to invade dentinal tubules[40]. It was suggested that bacterial cells might preferentially bind to immobilized molecules.

Results of the experiment I indicated that *E. faecalis* A197A, when grown at 46°C, bound significantly more to collagen type I, but not to BSA. This observation for the strain A197A is in line with those reported for other strains in terms of conditional adherence[42,56,88]. It is deduced from the results of the experiment I that growth at 46°C is more promoting in terms of binding to collagen than growth at pH 8.5. While 46°C is not a physiological temperature or one which is likely to affect growth conditions in the root canal, it is representative of a stressful condition. With the finding that various stress conditions can induce production of special as well as common proteins[18,58] one may speculate that other stress conditions may trigger similar responses by *E. faecalis*. Furthermore, it has been shown that even under physiological conditions, not strictly at 46°C, the bacterial collagen adhesin Ace can be produced[43]. In the serum of patients with *E. faecalis* endocarditis, anti-Ace antibodies were present suggesting that Ace could be produced during human infections[43]. Then, it may also be produced during endodontic infections.

In experiment I, testing a range of pH levels, we could not observe an increase in binding to collagen to the extent the bacterium performed after growth at 46°C. However, there was still a pronounced increase in binding to collagen when the bacterium was grown at mildly alkaline media. As this experiment has evaluated the effect of a limited range of pH which allowed the

growth of the species, the effect of exposure to higher pH levels on the adhesiveness of the bacterium deserves further investigation.

The major finding in experiment II was that cells of *E faecalis* pre-treated with collagen survived the calcium hydroxide challenge better than those not pre-treated with collagen. However, this was noticeable only at the 12 h period. On the other hand, the presence of collagen in the challenge medium did not inhibit the antimicrobial effect of calcium hydroxide, as no difference was observed between groups 2 and 3, where collagen was present in one and absent in the other.

We assume that the bacterial cells in suspension adhered to the collagen molecules as a result of pre-treatment of the bacteria with the collagen. This presumption relies on our pilot experiments where reduced adherence of *E. faecalis* to immobilized collagen was observed following pre-treatment of the bacteria with soluble collagen (Fig. 27).

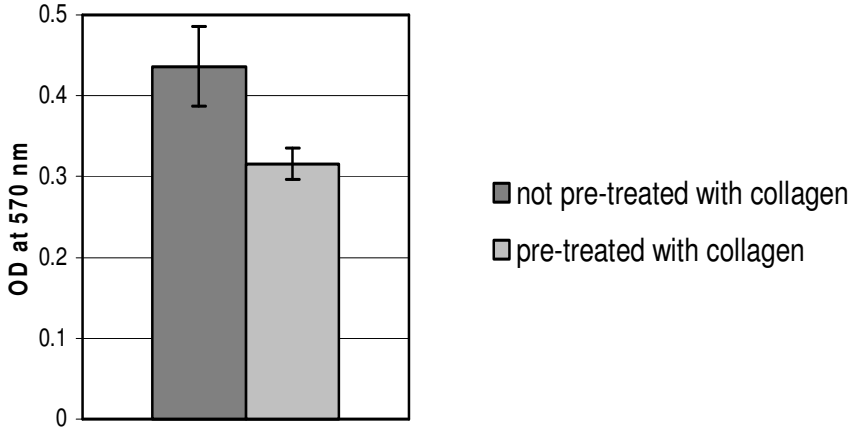

Figure 27 Effect of pre-treatment with soluble collagen on the adhesion of 46°C grown cells to collagen-coated surfaces as evaluated by the crystal violet assay

The experiment II was performed using bacteria grown at 46°C, since *E. faecalis* A197A binds to collagen significantly more following growth at 46°C. However, it is possible that other stresses, such as limited nutrient environment, toxins of other species or root canal medicaments, may act similar and increase the collagen adherence of the bacterium, which results in decreased susceptibility to calcium hydroxide.

In experiment II, the protective effect of adherence to collagen by the bacteria was observed as a temporary delay of killing by calcium hydroxide. However, it is possible that this effect might prevail for longer periods in the root canal system where the antimicrobial effect of calcium hydroxide is reduced, probably through buffering by dentine[21,52]. Probably it is the hydroxylapatite component of the dentine, not collagen, which buffers the antimicrobial effect of calcium hydroxide, since the presence of collagen in the present study (Group 2) did not inhibit the effect of calcium hydroxide in experiment II.

As a result of the observation that free collagen prevented tubule invasion by streptococci, it was suggested that soluble collagen preparations or collagen analogs could be useful in preventing endodontic streptococcal infections[38]. However, it must be taken into consideration that bacteria, at least *E. faecalis*, can become more resistant to subsequent treatments if they are let to bind to collagen.

The major finding in experiment III was that cells of *E faecalis* grown at 46°C survived the calcium hydroxide challenge longer than those grown

at 37°C. The results of the experiment III indicated that growth of bacteria at a stressful condition directly conferred resistance to the bacteria against calcium hydroxide, through a mechanism distinct from that seen in the experiment II.

While a number of studies tested the sensitivity of *E. faecalis* to lethal challenges following brief pre-treatment durations (from 5 sec to 30 min) with certain stresses[9,12,13,15], others applied prolonged pre-treatment durations (from 3 h to 24 h)[12,17]. Both pre-treatment procedures rendered the bacteria more resistant to subsequent challenges in most cases. However, prolonged pre-treatment of the bacteria with the sub-lethal condition proved to be more effective in terms of development of tolerance by bacteria[12,17]. In experiment III, bacteria were maintained under a prolonged stressful condition, and distinct from previous studies exposure was throughout growth (log phase). Exposure of the growing bacteria to the stress was terminated at the onset of the stationary phase. Thus, the pure effect of 46°C as a stressful condition could be observed, since other stresses, namely carbohydrate starvation, would be pronounced if the culture was allowed to progress to the stationary phase. Compared with exponential phase cells, starved bacteria were previously shown to be more resistant to ethanol, acid, heat and oxidative challenge[17] and to endodontic disinfectants[54].

Like in experiment I and in experiment II, 46°C was considered as a representative of a stressful condition also in experiment III. Bacteria may face a variety of stressful conditions in the root canal which prepare them for subsequent challenges. The basis of this assumption comes from previous studies where increased resistance to lethal challenges was observed following brief exposure to

sub-lethal doses or following starvation[9,12-15,17,23,54]. By analogy, bacterial cells growing at the limited-nutrient environment of the root canal may become less susceptible to root canal medicaments. Also, once the bacterium survives any medicament delivered into the root canal, it may become less susceptible to subsequent treatments.

Following a sudden increase in temperature, all cells –from the simplest prokaryotic cell to the most highly differentiated eukaryotic cell- increase production of certain molecules that protect them from harm. When this phenomenon was first observed, it was called the heat-shock response. However, subsequent studies revealed that the same response occurred when the cells were exposed to a variety of other challenges. For example, it occurs in traumatized cells growing in culture, in the tissues of feverish children, in the organs of heart-attack patients or cancer patients receiving chemotherapy[86]. Because, other than increase in temperature, diverse environmental challenges were responded similarly by the cell, the term 'heat-shock response' was replaced with 'stress response' and this universal response to adverse conditions was considered as a basic cellular defence mechanism[86]. It is possible that a stress response was triggered by the bacteria during growth at 46°C and transition to a self-protective state might have protected the cells from killing by the lethal effects of calcium hydroxide.

It may also be the mere presence of Ace (the *E. faecalis* collagen adhesin produced during growth at 46°C) on the cell surface of the 46°C-grown bacteria that protected them from killing by calcium hydroxide. The adhesin

produced on the surface of the 46°C-grown bacteria, but not on the 37°C-grown bacteria, may have brought about thickening of the bacterial cell wall and thus constituted a sort of barrier against the hydroxyl ions of calcium hydroxide acting to diffuse into the bacterial cytoplasm.

According to the findings in these experiments and previous studies an endodontic disease model has been designed (Figs 28 and 29). This model presents the effects on cells of *E. faecalis* of various pH levels occurring following delivery of calcium hydroxide into the root canal. The consequences of exposure to a sub-lethal pH may be acquisition of tolerance by the stress-facing bacterium (pH 10.0–pH 11.0), growth and generation of stronger lines (pH 9.0-pH 9.5) or increased adhesion to collagen (pH 8.0-pH 8.5) which is associated with increased dentinal tubule invasion and decreased susceptibility to lethal challenges.

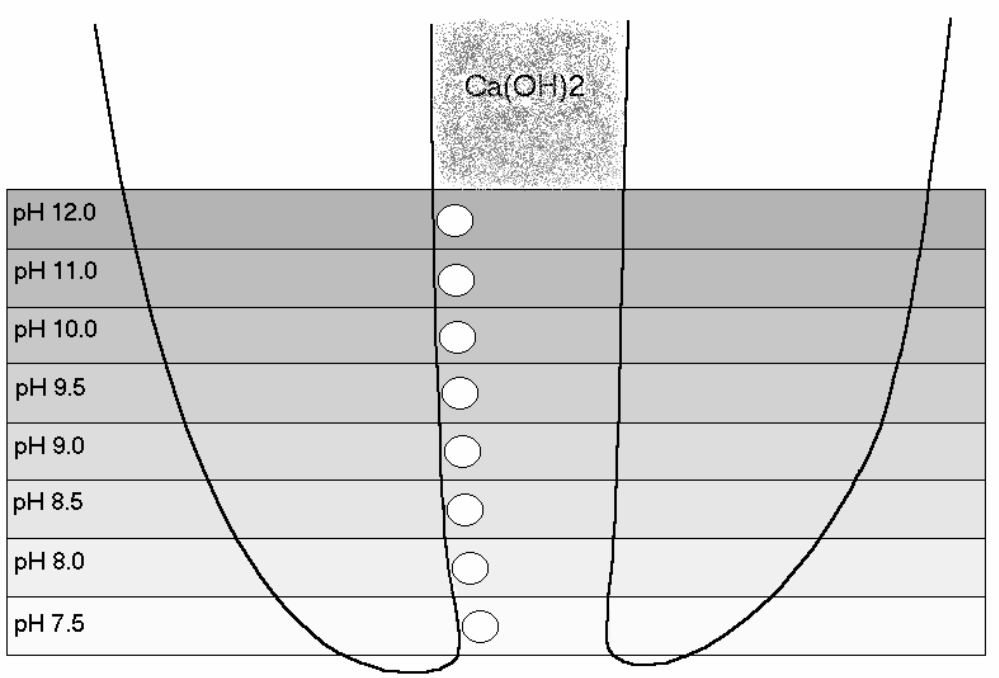

Figure 28 Following delivery of calcium hydroxide into the root canal, a pH gradient occurs along the root canal which exerts various effects on cells of *E. faecalis* residing on the root canal surface

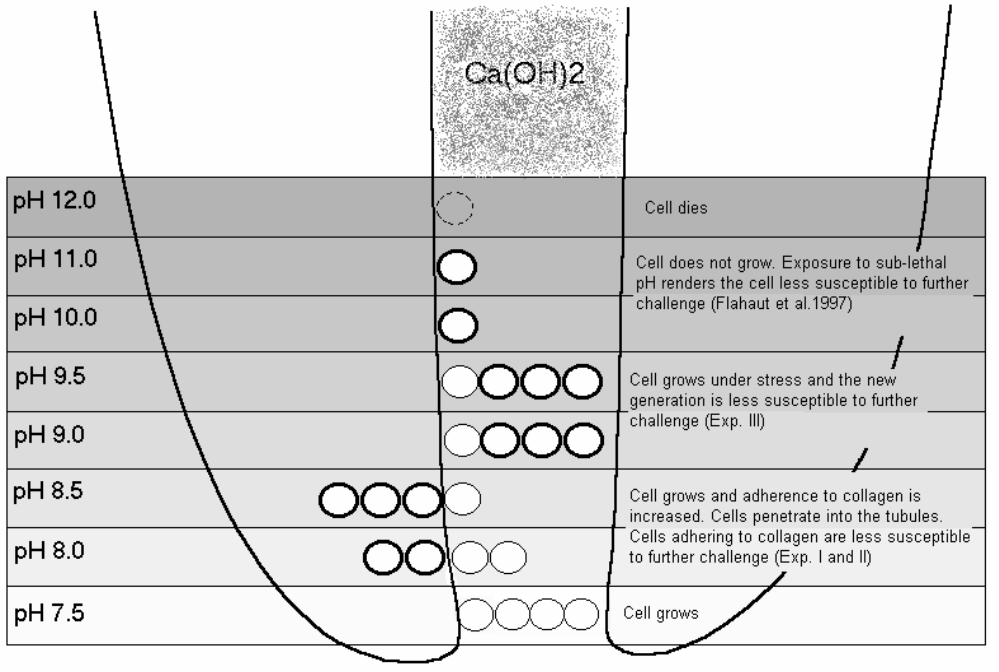

Figure 29 Assumptional effects of various pH levels on cells of *E. faecalis*.

The data obtained from the experiments designed as experiment I, experiment II and experiment III support the hypothesis related to the infection and survival mechanisms held by *E. faecalis* (Fig.1) and provide new insights into the persistence of *E. faecalis* in endodontic infections. According to the experiment I, *E. faecalis* A197A adhered to the collagen-coated surfaces more following growth at slightly alkaline media (pH 8.0 and pH 8.5) and following growth at 46°C. A growth medium of pH 8.5 and a growth temperature of 46°C were also shown to be stressful for the bacterial growth (Fig 9 and 12). The results of the experiment II suggested that cells of *E. faecalis* binding to collagen in the suspension form were less susceptible to challenge with calcium hydroxide at one period (at 12h). The results of the experiment III indicated that growth at a stressful condition, represented by growth at 46°C, rendered the cells of *E. faecalis* A197A less susceptible to challenge with calcium hydroxide.

To current knowledge, elimination of *E. faecalis* in the root canal system seems to be difficult. Therefore interfering with the initial attachment of the bacterium to the walls of the dentinal tubules may be a means to limit the bacterial establishment in the dentinal tubules. A possible preventive measure may be disruption of the dentinal collagen, the target for the bacterial adhesins. Enzymatic modulation is one possible way of altering the collagen. Similarly, proteinaceous bacterial adhesins may be targeted by protein digesting agents such as trypsin. These methods have been tested *in vitro* and resulted in decreased adherence of *E. faecalis* to collagen-coated surfaces[88].

For the antibacterial applications against *E. faecalis*, the use of sodium hypochlorite (NaOCl), chlorhexidine (CHX), iodine potassium iodide (IKI) and camphorated paramonochlorophenol (CMCP) can be recommended, but not calcium hydroxide. Previous studies have shown the ineffectiveness of calcium hydroxide in the elimination of *E. faecalis* in experimentally infected root specimens[20,46] and in the root canals of animals[76]. However, complete disinfection was achieved with the use of CMCP in these experiments. Mixture of calcium hydroxide with CMCP also was suggested to perform better than calcium hydroxide mixed with saline[70,77]. Sodium hypochlorite, CHX and IKI were also shown to significantly reduce the viable numbers of *E. faecalis* in experimentally infected root specimens[24,74]. Moreover, it was suggested that mixture of calcium hydroxide with NaOCl, CHX or IKI disinfected the dentinal blocks infected with *E. faecalis* more effectively than mixture of calcium hydroxide with water[74,90]. Furthermore, dentine powder models, where root canal medicaments were pre-treated with ground dentin before exposure to bacteria, have indicated successful killing of *E. faecalis* with NaOCl (1 %), CHX (0.5 %) and IKI (2/4 %)[21]. However, the antimicrobial activity of calcium hydroxide was abolished after pre-treatment with organic substances such as, dentine powder, hydroxylapatite or bovine serum albumin[21,52]. It is also recommendable that root canal medicaments be used at sufficient concentrations and for extended durations for the efficient killing of *E. faecalis*. Otherwise, surviving bacteria are likely to grow tougher and more virulent, and will be much harder to eliminate in the next visit.

In this work, a part of the infection and survival mechanisms held by *E. faecalis* was presented. However, these findings should also be tested in experimental designs where dentine and the other related host factors are included. The infection and survival mechanisms of *E. faecalis* is a vast topic and includes certainly more than what is presented here. Further research in this field is required.

CONCLUSION

Stressful growth conditions, including increase in pH which is a consequence of treatment with alkaline medicaments such as calcium hydroxide, increases the collagen binding ability of *E. faecalis* A197A, and may facilitate dentinal tubule invasion of this species.

While the presence of collagen itself does not exert inhibitory effect on calcium hydroxide, cells of *E. faecalis* A197A binding to collagen appear to be more resistant to the antimicrobial effect of calcium hydroxide.

Growth of *E. faecalis* A197A at a stressful condition, as represented by growth at 46°C, increases the resistance of the bacterium to challenge with calcium hydroxide.

These can be critical mechanisms by which *E. faecalis* survives calcium hydroxide medications and predominates in persistent endodontic infections.

SUMMARY

Enterococcus faecalis is a Gram-positive facultative anaerobic bacterium and is known to tolerate a broad range of harsh conditions. It can cause a variety of diseases in man including endodontic infections. It is often recovered from obturated root canals exhibiting signs of apical periodontitis.

While most of our knowledge about the pathogenicity of *E. faecalis* comes from the medical literature, details about the infection and survival mechanisms held by *E. faecalis* in the root canal is scarce.

In this work, we aimed to explain how *E. faecalis* survives calcium hydroxide (a commonly used and a highly alkaline endodontic disinfectant) and to show that growth of the bacterium at stressful conditions, including growth at alkaline broth, can increase its ability to adhere to collagen.

Three experiments were designed. In experiment I, *E. faecalis* A197A was grown in broth of adjusted pHs varying between 7.1 and 9.5, and also in a broth of 7.1, but at 46°C as the growth temperature (an established stressful growth condition). Aliquots of bacterial suspensions were added to wells coated either with bovine serum albumin or with collagen type I. Bacteria adhering to the surfaces were stained with crystal violet. Following dissolution of the stain the number of bacteria adhering to the surfaces was quantified spectrophotometrically.

In experiment II, *E. faecalis* A197A grown at 46°C were pre-treated either with collagen type I or with PBS for an hour. The bacteria were then

challenged with saturated solution of calcium hydroxide. Samples were drawn at 1h, 6h, 12h and 24h and the surviving bacteria were counted.

In experiment III, *E. faecalis* A197A was grown at 37°C or 46°C, and challenged with the saturated solution of calcium hydroxide. Samples were drawn at 1h, 6h, 12h, 18 h and 24h and the surviving bacteria were counted.

The results of the experiment I indicated that the adhesion of *E. faecalis* A197A to BSA-coated surfaces decreased inversely with an increase in alkalinity of the medium. Contrarily, the adhesion to collagen type I-coated surfaces increased with alkalinity of the medium, reaching a peak after growth at pH 8.5. Furthermore, bacteria grown at 46°C adhered to collagen more than any group.

The results of the experiment II indicated that *E. faecalis* A197A adhering to collagen, as a result of pre-treatment with collagen, survived the calcium hydroxide challenge better than the control groups at 12h.

The results of the experiment III indicated that bacteria grown at 46°C survived the calcium hydroxide challenge better than bacteria grown at 37°C, at 6h, 12h and 18h.

These results suggest that stressful growth conditions, including increase in environmental pH- which is a consequence of treatment with alkaline medicaments such as calcium hydroxide, increases the collagen binding ability of *E. faecalis* A197A and may facilitate dentinal tubule invasion of this species. Adhesion to collagen, furthermore, confers the cells of *E. faecalis* A197A resistance against calcium hydroxide. On the other hand, through stressful growth

(as represented by growth at 46°C) a tough generation of bacteria can be developed which is more resistant to calcium hydroxide than bacterial cells grown at optimum conditions.

These may be critical mechanisms by which *E. faecalis* predominates in persistent endodontic infections.

ÖZET

Endodontik Hastalık Bakımından *Enterococcus faecalis*'in Enfeksiyon ve Direnç Mekanizmalarının İncelenmesi

Enterokoklar insanda normal florada komensal olarak gastrointestinal sistem, oral kavite ve vajinada bulunurlar. Bununla birlikte idrar yollarını, kan dolaşım yollarını, safra yollarını, endokardiumu, karnı, yanık yaralarını ve kateter gibi vücuda yerleştirilen yabancı cisimleri enfekte ederek ciddi hastalıklara yol açabilmektedirler[28]. Enterokokların endodontik enfeksiyonlarda da rol oynadıkları bilinmektedir. *Enterococcus faecalis*, tedavi edilmemiş nekrotik pulpalı dişlerin mikrobiyal florasının küçük bir kısmını oluşturmaktadır[3,60,71,80]. Buna karşın, kronik apikal periodontitis bulgusu veren başarısız endodontik tedavili dişlerin kök kanallarının pozitif kültürlerinin %30-70'inde izole edilmiş ve sıklıkla da saf kültür halinde bulunmuştur[19,22,48,51,81]. Moleküler çalışmalar da başarısız endodontik tedavili dişlerin %60-90'ında *E. faecalis*'e ait genetik materyal saptamıştır[19,60,61,66,72]. *E. faecalis*'in antimikrobiyal kök kanal ilaçlarının kullanıldığı endodontik tedaviye nasıl direnç gösterdiği ve enfeksiyonu devam ettirdiği konusunda çok az şey bilinmektedir. Bu tezin amacı *E. faecalis*'in enfeksiyon ve hayatta kalma mekanizmalarının daha iyi anlaşılmasına katkıda bulunmaktır.

Kök kanal sisteminde mikroorganizmaların optimum olmayan şartlar ve stres altında hayatlarını devam ettirdikleri düşünülebilir. Bunun yanı sıra endodontik tedavi sırasında uygulanan kök kanal ilaçları ilave bir stres ortamı oluşturur. Böyle bir ortamda yaşam ve çoğalma bakterinin direncini doğrudan

arttırabilir veya stres ortamı bakterinin kollajene bağlanmasını stimüle edebilir. Kollajene bağlanan bakteri antimikrobiyal ajanlara karşı dolaylı yoldan da direnç kazanabilir. Bu hipotez Fig. 1'de gösterilmektedir ve bu tez çalışmasının temelini oluşturmaktadır.

Enterokoklar zor çevresel şartlara iyi dayanıklılık gösterirler. Enterokoklar 10°C ve 45°C arasında, pH 9.6 değerinde veya %6.5 NaCl besiyerinde çoğalabilmekte, 60°C'de 30 dakika yaşayabilmektedirler[67]. Öldürücü olmayan fiziksel veya kimyasal etkenlere maruz bırakılmasının ardından *E. faecalis*'in, normalde öldürücü dozlardaki sodyum dodesil sülfat, safra tuzları, hiperozmolarite, ısı, etanol, hidrojen peroksit, asidite ve alkalenite seviyelerine daha az hassas hale geldiği bulunmuş ve hatta farklı etkenlere karşı çapraz olarak korunduğu gösterilmiştir[12-15]. Bunun yanı sıra besinden yoksun *E. faecalis*'in uzun süreler boyunca canlılığını devam ettirebildiği ve ısı, hidrojen peroksit, etanol ve asidin yanısıra sodyum hipoklorit ve UV irradyasyonuna karşı da dirençli hale geldiği tespit edilmiştir[17,23].

E. faecalis'in dentin tübüllerine penetre olabildiği *in vitro* çalışmalarla gösterilmiştir[1,20,39,46,50,85]. Bunun yanı sıra *E. faecalis*'in kök kanal dezenfektanı olarak günümüzde sıklıkla kullanılan kalsiyum hidroksite dirençli olduğu gözlenmiştir [4,7,20,46,70,74,76,85].

Kök kanal sisteminin mikrobiyal çoğalma açısından besinden zengin olduğu düşünülmemekle birlikte *E. faecalis*'in apikal foramenden kök kanalı içerisine sızan serumu kullanarak çoğalabileceği düşünülmektedir[11,39]. Diğer taraftan sahip olduğu virülans faktörlerinden hyalüronidaz aracılığıyla

dentinde bulunan hyalüronattan [5,27,29,31] veya organik kısımca zengin olduğu bilinen smear tabakasından[83] çoğalmak için gerekli enerjiyi elde edebileceği düşünülmektedir.

Mikroorganizmaların konakçı dokuya adhezyonu çoğu enfeksiyöz hastalık için ilk basamaktır. *E. faecalis*'in, kollajen tip I dahil, ekstraselüler matriks proteinlerine adhezyonu önceki çalışmalarla gösterilmiştir [39,42,43,56,62,88,89]. Bakterilerin kollajen tip I'e adhezyonu, bunun dentinin ana organik komponenti olmasından dolayı önemlidir[36]. Yapılan çalışmalarda çeşitli *E. faecalis* izolatlarının 46°C'de çoğaltıldıklarında (bu bir stres ortamını temsil etmektedir) ekstraselüler matriks proteinlerine adhezyonlarının arttığı gösterilmiş ve bu olay 'kondisyonel adherans' olarak adlandırılmıştır[88]. Daha sonra yapılan araştırmalarda *E. faecalis*'in 46°C'de çoğaltıldığında kollajene yaptığı bu artan bağlanmayı 'Ace' adı verilen bir adhezin üreterek başardığı anlaşılmıştır[42,43,56]. Bir endodontik enfeksiyondan elde edilmiş *E. faecalis* A197A kökeni[73] ile yaptığımız pilot deneyler bakterinin kollajen tip I'e kondisyonel adherans yaptığını gösterdi (A197A kökeni Ace adhezinini eksprese edecek gene sahiptir[55]). Bunun üzerine, kök kanallarında görülebilecek diğer stres ortamlarının da bakterinin organik moleküllere bağlanmasına etki edebileceği düşünüldü. Dentin tübüllerinde *E. faecalis*'i elimine etmedeki yetersizliğini göz önüne alarak kalsiyum hidroksitin meydana getireceği alkalenitenin bakteri adhezyonunu ne şekilde etkileyebileceğini araştırmak istedik. Deney I'in amacı bakteriyel çoğalma ortamı olarak pH 7.1 ile 9.5 arasındaki pH değerlerinin bakterinin organik yüzeylere (kollajen tip I ve bovin serum albumin) adhezyonuna etkisinin

incelenmesi ve bu pH gruplarıyla elde edilen sonuçların 46°C ile elde edilen sonuçlarla karşılaştırılmasıydı.

Deney I'de normal pH değeri 7.1 olan tryptone soya besiyerine (TSB) NaOH ilavesiyle pH değeri 7.5, 8.0, 8.5, 9.0 ve 9.5 olan besiyerleri hazırlandı. Bir endodontik enfeksiyondan izole edilmiş olan *E. faecalis* A197A kökeni[73] besiyerlerine inoküle edilerek 37°C'de enkübe edildi; pH değeri 7.1 olan besiyerinin bir kısmı ise 46°C'de enkübe edildi. Bakteriler yıkanarak PBS içinde resüspande edildi.

Bakteriler üzerinde hangi ortamın stres oluşturduğunun saptanması amacıyla çoğalma eğrileri spektrofotometre yardımıyla belirlendi.

Bakteri adhezyonu 96-gözlü mikrotiter plaklarında test edildi. Gözler bovin serum albumin veya kollajen tip I ile bir gece önceden kaplandı. Yoğunlukları spektrofotometrik olarak standardize edilen bakterilerin eşit miktardaki süspansiyonları gözlere yerleştirildi. İki saatlik enkübasyon süresinin ardından duvarlara bağlanmış bakteriler kristal violet ile boyandılar. Her bir göze ait boyanma derecesi hücre tarafından abzorbe edilmiş kristal violet maddesinin etanol-aseton ile çözülmesi ve çözünen boyanın spektrofotometrik olarak okunmasıyla saptandı.

Spektrofotometrik değerlerin gerçekte ne kadar bakteriye denk geldiği daha önceden hazırlanmış eğriler yardımıyla hesaplandı (Fig. 2 ve 3). Veriler, istatistiksel anlamlılık değeri $\alpha=0.05$ alınarak Kolmogorov-Smirnov testi, Levene's test ve Student's *t*-testi ile değerlendirildi.

Bakteriyel adhezyon karakteristikleri aynı zamanda SEM ile de incelendi.

Her gruba ait çoğalma eğrileri Fig. 6-12 de gösterilmektedir. Buna göre tüm gruplarda bakteri çoğalması gerçekleşmiş olmakla beraber, pH 8.5, pH 9.0 ve pH 9.5 besiyerlerinin ve ayrıca 46°C olan çoğalma sıcaklığının bakteriler için stres ortamı oluşturduğu anlaşıldı.

Adhezyon deneyinin sonuçları Fig. 13 ve 14'te gösterilmektedir. Buna göre BSA kaplı yüzeylere bakteriyel adhezyon pH 7.1 grubundan pH 9.5 grubuna doğru giderek azaldı. pH 7.1 ortamında çoğalan bakteriler BSA'ya tüm gruplardan daha fazla adhezyon gösterdi. Diğer taraftan 46°C'de çoğalan bakteriler ile 37°C'de çoğalan bakterilerin BSA'ya bağlanmaları arasında belirgin bir fark yoktu.

Kollajen kaplı yüzeylere bakteriyel adhezyon pH 7.1 grubundan pH 8.5 grubuna kadar artış gösterdi. Farklı pH grupları arasında en yüksek bağlanma pH 8.5 grubunda gözlendi. pH 8.0 ve pH 8.5 grupları pH 7.1, pH 9.0 ve pH 9.5 gruplarından anlamlı olarak daha fazla bağlanma gösterdi. pH 9.0 ve pH 9.5 grupları kollajene bağlanma bakımından pH 7.1 grubuyla karşılaştırıldığında anlamlı farklılık gözlenmedi. Diğer taraftan 46°C'de çoğalan bakteriler tüm gruplardan açık bir şekilde daha fazla bağlanma gösterdi.

Her bir grubun kendi içinde kollajen ve BSA'ya bağlanması karşılaştırıldığında pH 7.1 (37°C) grubu dışındaki tüm gruplarda bakterilerin kollajene bağlanmaları BSA'ya bağlanmalarından anlamlı olarak daha yüksek bulundu.

Bakteri adhezyonunu gösteren SEM fotoğrafları Fig.15-24'te görülmektedir. Genel olarak, kollajen kaplı yüzeye bağlanan hücreler yüzeye eşit olarak dağılmış hücre kümelerinden oluşmakla birlikte (Fig. 15,16,20-22), BSA kaplı yüzeye bağlanan hücreler daha düzensiz dağılım göstermiş ve yüzey üzerinde şeritler oluşturmuştur (Fig. 17-19,23,24).

E. faecalis'in dentin tübül invazyonu yaptığı gösterilmiş olmakla birlikte[1,20,39,46,50,85] bu yeteneğin bakterinin kollajen adhezyonu ile ilgili olduğu bildirilmiştir[39]. *E. faecalis*'in kollajene bağlanmasını sağlayan 'agregasyon substansı' ve 'Ace' adlı protein yapıda iki adhezini bilinmektedir[56,62]. Bunlardan Ace en dikkat çekeni olup bakteri 46°C'de çoğaltıldığında eksprese edilmektedir. Ace adlı adhezinin bakterinin dentine bağlanmasındaki önemi son zamanlarda yapılan çalışmalarla gösterilmiştir[25,33]. Bu deneyde kullanılan A197A kökeni Ace adhezinini üretebilecek *ace* genine sahiptir[55]. Bu deneyde 46°C'de çoğaltılan grubun kollajene gösterdiği artan bağlanmanın Ace adhezini aracılığıyla gerçekleştiği düşünülmektedir.

Diğer taraftan, bakteri hücresi yüzey gerilimi, elektrik yükü ve hidrofobisitesi gibi fizikokimyasal özellikleri de adhezyonda etkili olmaktadır. Ortam pH'sındaki değişimler bu parametreleri etkileyebilmektedir[45]. Dolayısıyla bakterinin kollajene artan bağlanmasında spesifik adhezinlerin yanı sıra fizikokimyasal özelliklerinde meydana gelen değişiklikler de etki göstermiş olabilir. Bununla birlikte, bu deneyde pH 8.0 ve pH 8.5 gruplarının artan kollajen bağlanmalarını ne şekilde sağladıkları bilinmemektedir.

Kalsiyum hidroksit 12.5'e varan pH değeriyle kuvvetli bir kök kanal dezenfektanıdır. Ancak kök kanalı içerisinde kalsiyum hidroksitin gönderilmesi ile ilgili problemlere[6,59,69], farklı formülasyonlarının alkalenizasyon potansiyelleri ile ilgili varyasyonlara[64], dentinde dağılımı ile ilgili varyasyonlara[44,84] ve dentin tarafından etkisinin tamponlaması[21,52] gibi faktörlere bağlı olarak öldürücü pH'a her zaman ulaşılamamaktadır. Bunun sonucunda hafif alkalen ortamda çoğalan *E. faecalis* hücrelerinin kollajene artan adhezyon yetenekleri ile dentin tübül penetrasyonlarını arttırarak enfeksiyon derinliğini fazlalaştırabilecekleri anlaşılmıştır. Artan tübül penetrasyonu sonucunda daha derine yerleşen bakteri hücreleri de daha sonraki dezenfektan uygulamalarından daha az etkileneceklerdir.

Albumin, bir doku sıvısı olan serumun ana komponentidir ve kök kanalı içerisine periodontal dokulardan serum sızıntısı meydana gelebilmektedir. Bakteri adhezyonunun hafif alkalen ortamlarda BSA'dan çok kollajene olması, alkalen medikaman uygulamasının ardından bakterinin serum içerisindeki albümin ile bloke olmayıp kollajene bağlanmayı tercih edebileceğini göstermektedir.

Deney I'de kullandığımız *E. faecalis* A197A kökeninin 46°C'de çoğaltılmasıyla kollajene gösterdiği yüksek bağlanma, 'kondisyonel adherans' olarak adlandırılan ve diğer *E. faecalis* izolatları ile gösterilen durum ile uyumludur[42,56,88]. 46°C insan vücudunda görülen bir sıcaklık olmamasına karşın bir stres ortamını temsil eder. Farklı stres ortamlarının bazı spesifik proteinler kadar genel ve ortak proteinlerin de sentezini teşvik edebildiği[18,58] bulgusundan

yola çıkarak diğer stres ortamlarının da *E. faecalis* hücreleri üzerinde benzer etki göstermeleri beklenebilir. Bununla birlikte, yapılan araştırmalar kollajen adhezini Ace'nin yalnızca 46°C'de değil, fizyolojik şartlarda da bakteri tarafından üretilebildiğini göstermektedir[43]. *E. faecalis* kaynaklı endokardit hastalarının serumlarında Anti-Ace antikorlarının saptanmış olması Ace'nin insan enfeksiyonlarında da görülebileceğini ortaya koymaktadır[43]. Dolayısıyla, kuvvetli bir adhezin olan Ace'nin endodontik enfeksiyonlarda da etkili olabileceği düşünülmektedir. Bu deneyde, bakteriler üzerinde bir stres ortamı oluşturduğu gösterilen pH 8.5 çoğalma ortamı da 46°C'lik çoğalma ortamı kadar olmasa da bakterinin kollajene bağlanmasını belirgin şekilde arttırmıştır.

E. faecalis üzerinde kalsiyum hidroksitin öldürücülüğünün test edildiği çalışmalarda dentin maddesi dahil edilmediğinde *E. faecalis*'in kolayca elimine edildiği bildirilmiştir[8]. Buna karşın, dentinin dahil edildiği deney dizaynlarında (bu, gerçek şartlara daha yakın olan deney dizaynıdır), *E. faecalis* elimine edilememiştir[20,21,46,52,70,74,85]. Kollajen tip I'in dentinin ana organik komponenti olmasına dayanarak *E. faecalis*'in kollajene adhezyonunun kalsiyum hidroksite karşı direnç ile ilişkisinin incelenmesi Deney II'nin amacıydı.

E. faecalis A197A kökeni[73] TSB içerisinde 46°C'de erken plato fazına kadar çoğaltıldı ve yıkanarak PBS içinde resüspande edildi. Bakterinin eşit miktarları 3 gruba ayrılarak test edildi. Grup 1'de bakteriler 1 saat süresince kollajen ile muamele edildikten sonra kalsiyum hidroksitin sulu çözeltisine maruz bırakıldı. Grup 2'de kollajen ile muamele yapılmadı; ancak kollajen, kalsiyum hidroksitin sulu çözeltisiyle eş zamanda bakteri süspansiyonu üzerine eklendi.

Grup 3'te kollajen hiç yer almadı; sadece kalsiyum hidroksitin sulu çözeltisi bakteri süspansiyonu üzerine eklendi. Deney düzeni Fig. 4 ve 5'te gösterilmektedir. Kalsiyum hidroksitin eklenmesinden 1, 6, 12 ve 24 saat sonra örnekler alınarak canlı bakteri sayımı yapıldı. Veriler, istatistiksel anlamlılık değeri α=0.01 alınarak Student's *t-testi* ile değerlendirildi.

Deney II'nin sonucu Fig. 25'te gösterilmektedir. Buna göre ilk 6 saatte hiçbir grupta canlı bakteri sayısında azalma olmadı. Ancak 12. saatte Grup 1'de (kollajen ile muamele edilen bakteri grubunda) diğer gruplara göre anlamlı olarak daha fazla sayıda canlı bakteri bulundu. Grup 2 ve Grup 3 arasında anlamlı fark yoktu. 24. saatte hiçbir grupta canlı bakteri kültüre edilemedi.

Bu sonuçlar, kollajen ile muamele edilen bakterilerin kalsiyum hidroksite karşı geçici bir süre için dahi olsa daha yüksek direnç gösterdiğini ortaya koymaktadır. Diğer taraftan Grup 2 ve Grup 3 arasında fark olmayışı kollajenin kendisinin kalsiyum hidroksitin etkinliğini azaltmadığını göstermektedir.

Pilot deneylerimiz, 46°C'de çoğalan bakteri ile kollajenin PBS içerisinde 1 saat süresince muamele edilmelerinin sonucunda bakterinin kollajen kaplı yüzeylere adhezyonunun azaldığını göstermiştir; bu da bakterinin muamele sırasında kollajene bağlandığını göstermektedir (Fig. 27).

Deney II, 46°C'de çoğaltılan bakteriler ile gerçekleştirilmiştir; çünkü kollajene bağlanma potansiyeli en yüksek bakteriler, bilgimiz doğrultusunda, bu şekilde elde edilebilirdi. Bununla birlikte bakterinin kollajen adhezyonunu arttırması muhtemel diğer stres kaynakları da (besin yokluğu, diğer

mikroorganizmaların toksinleri, kök kanal dezenfektanları varlığı gibi) kök kanallarında mevcuttur.

Bu deneyde yalnızca 12. saatte görülen kollajene bağlanmanın koruyucu etkisi, kök kanallarında sürekli veya en azından daha uzun süreli olabilir. Çünkü, kalsiyum hidroksitin öldürücü etkisinin dentin tarafından azaltıldığı bilinmektedir[21,52]. Diğer taraftan, kalsiyum hidroksitin etkisini tamponlayan kısmın büyük ihtimalle dentinin hidroksilapatit kısmı olduğu düşünülmektedir; çünkü bu çalışmada kollajenin varlığı (Grup 2) ile yokluğu (Grup 3) arasında bir fark görülmedi.

Bakterinin stres altında çoğalmasının, kollajene bağlanmasını arttırarak kalsiyum hidroksite karşı direnç kazanmasını dolaylı yoldan sağlamasının yanı sıra, doğrudan direnç kazanmasına da yol açabileceği düşünülebilir. Daha önceki çalışmalar bir süre strese maruz kalan *E. faecalis* hücrelerinin toleranslarının arttığını göstermektedir[12-15]. Deney III'ün amacı stres altında çoğalan *E. faecalis* hücrelerinin kalsiyum hidroksite direncinin optimum şartlarda çoğalan hücrelerinki ile karşılaştırılmasıydı.

E. faecalis A197A kökeni[73] TSB içerisinde 46°C'de veya 37°C'de çoğaltıldı ve yıkanarak PBS içinde resüspande edildi. Kalsiyum hidroksitin sulu çözeltisine maruz bırakılan test gruplarından 1, 6, 12, 18 ve 24. saatlerde örnekler alındı ve canlı bakteri sayımı yapıldı. Veriler, istatistiksel anlamlılık değeri α=0.01 alınarak Mann Whitney U ve Wilcoxon testleri ile değerlendirildi.

Deney III'ün sonucu Fig. 26'da gösterilmektedir. Buna göre, 37°C'de çoğalan bakteriler ilk 1 saat kalsiyum hidroksitten etkilenmezken

46°C'de çoğalan bakteriler ilk 6 saat kalsiyum hidroksitten etkilenmediler. 6 ve 12. saatlerde istatistiksel olarak 46°C grubunda 37°C grubuna göre daha fazla canlı bakteri bulundu. 18.saatte 37°C grubunda canlı bakteri saptanamadı; bu nedenle 46°C grubu net bir şekilde daha dirençli bulundu. Bunun yanı sıra 46°C'de çoğalan bakteriler 24. saatte tamamen yok oldu.

Deney III'ün sonuçları, stres altında çoğalan *E. faecalis*'in kalsiyum hidroksite direncinin optimum şartlarda çoğalanınkinden daha fazla olduğunu göstermektedir. 46°C grubunda görülen bu direncin kaynağı olarak stres proteinleri[86] veya bakteri yüzeyinde eksprese edilen Ace adhezini düşünülmektedir. Bu adhezinin üretilmesiyle hücre duvarının kalınlaşarak dezenfektanın sitoplazmaya diffüzyonuna karşı bir çeşit bariyer görevi görebileceği tahmin edilmektedir.

Diğer deneylerde olduğu gibi Deney III'te de 46°C'lik çoğalma ortamı sıcaklığı bir stres ortamı temsilcisi olarak seçildi. Bununla birlikte kök kanalı içerisinde bakteriler daha başka stres ortamları ile karşılaşırlar ve bu streslerin de bakteriyi öldürücü etkilere karşı güçlendirme gibi rolü olabilir. Örneğin, kök kanalının sınırlı besin kaynağı ortamında çoğalabilen *E. faecalis* hücreleri daha sonra uygulanan kök kanal dezenfektanlarına daha dirençli hale gelebilirler. Veya benzer şekilde, bir kanal dezenfektanının öldürücü olmayan dozuna maruz kalarak çoğalan bakteri hücreleri bir dahaki sefere öldürücü doz ile karşılaştıklarında direnç gösterebilirler.

Bu deneylerin sonuçları ve önceki bilgiler birleştirilerek bir endodontik enfeksiyon modeli hazırlandı (Fig. 28, 29). Bu model kök kanalı

içerisine kalsiyum hidroksit gönderilmesiyle kök kanalının değişik seviyelerinde meydana gelen pH farklılıklarının *E. faecalis* hücreleri üzerindeki etkisini göstermektedir. Özetle: öldürücü olmayan pH ile karşılaşan bakteri direnç kazanabilir (pH 10.0-pH 11.0), çoğalma devam ederek bakterinin kendisinden daha dirençli jenerasyonlar meydana gelebilir (pH 9.0-pH 9.5) veya bakterinin kollajene bağlanması artarak, dentin tübül invazyon derinliği fazlalaşabilir (pH 8.0-pH 8.5). Kollajene bağlanması yoluyla ve daha zor ulaşılabilirliği sayesinde tübül derinliklerindeki bakterinin daha sonraki dezenfektan uygulamalarına karşı direnci artabilir (pH 8.0-pH 8.5).

Bu tez çalışmasında dizayn edilen 3 deneyin sonuçları *E. faecalis*'in enfeksiyon ve hayatta kalma mekanizmaları ile ilgili hipotezi (Fig. 1) desteklemektedir. Kısaca, deney I'de *E. faecalis* A197A hafif derecede alkalen ortamlarda (pH 8.0 ve pH 8.5) ve bunun yanı sıra 46°C'de çoğaldığında kollajene artan bağlanma gösterdi. Çoğalma ortamı olarak pH 8.5'in ve 46°C'nin stres ortamları oluşturduğu gösterildi. Deney II'de kollajene bağlanan *E. faecalis* A197A'nın kalsiyum hidroksite belirli bir periyotta (12. saat) daha dirençli olduğu bulundu (dolaylı yoldan direnç). Deney III'te ise 46°C ile temsil edilen stres ortamında çoğalan *E. faecalis* A197A'nın kalsiyum hidroksite daha dirençli olduğu gösterildi (doğrudan direnç).

Şu an ki bilgiye göre kök kanal sistemine yerleşmiş *E. faecalis*'in elimine edilmesi güçtür. Dolayısıyla, *E. faecalis* enfeksiyonu kök kanal sistemine daha yerleşmeden bakterinin dentin yüzeylerine erken adhezyonunun önlenmesi önemli bir koruyucu yöntem olabilir. Bu amaca yönelik bir yaklaşım dentin

kollajeninin enzimatik yollarla yapısının değiştirilmesi olabilir. Yine benzer şekilde protein yapıdaki bakteri adhezinleri, tripsin gibi protein sindirici enzimlerle hedef alınabilir. Bu yöntemler *in vitro* olarak test edilmiş ve *E. faecalis*'in kollajen kaplı yüzeylere bağlanmasını azaltmıştır[88]. *E. faecalis*'in kollajene bağlanmasının engellenmesiyle dentin tübül invazyonu önlenebileceği gibi bakterinin kollajene bağlanma yoluyla kök kanal dezenfektanlarına karşı kazanabileceği direncin de önlenebileceği düşünülmektedir.

Kök kanallarında *E. faecalis*'e karşı yapılan antimikrobiyal uygulamalarda kalsiyum hidroksit yerine sodyum hipoklorit (NaOCl), klorheksidin (CHX), iyodin potasyum iyodid (IKI) ve kafurlu paramonoklorfenolün (CMCP) kullanılması önerilebilir. Daha önceki çalışmalar, standardize dentin blokları[20,46] veya hayvanların kök kanallarında[76] kalsiyum hidroksitin *E. faecalis*'e karşı etkisiz kaldığını göstermektedir. Diğer taraftan, bu çalışmalarda dentinin tamamen dezenfeksiyonu CMCP kullanımıyla sağlanmıştır. Alternatif olarak, kalsiyum hidroksitin CMCP ile karıştırılarak uygulanmasının kalsiyum hidroksitin salin ile karıştırılmasından daha iyi dezenfeksiyon sağladığı bildirilmiştir[70,77]. Yine NaOCl CHX ve IKI'nın deneysel olarak enfekte edilmiş dentinde canlı *E. faecalis* hücre sayısını belirgin şekilde azalttığı gösterilmiştir[24,74]. Diğer çalışmalarda kalsiyum hidroksitin NaOCl, CHX veya IKI ile olan karışımlarının *E. faecalis* ile enfekte dentin bloklarında su ile olan karışımından daha etkili dezenfeksiyon sağladığı gösterilmiştir[74,90]. Dentin tozu modellerinde ise yine NaOCl (%1), CHX (%0.5) ve IKI (%2/4) ile *E. faecalis*'e karşı iyi dezenfeksiyon sağlandığı[21], buna karşın kalsiyum hidroksitin

antimikrobiyal etkisinin dentin tozu, hidroksilapatit veya bovin serum albumini ile ortadan kaldırıldığı gösterilmiştir[21,52]. Bunların yanı sıra, *E. faecalis*'in etkili bir şekilde öldürülebilmesi için kök kanal medikamanlarının yeterli konsantrasyonlarda ve mümkün olduğunca uzun sürelerde dentinle teması olacak şekilde kullanılmaları önerilebilir. Aksi halde hayatta kalan bakteriler daha dirençli şekilde çoğalacak ve bir daha ki sefere uygun dozlar kullanılsa bile öldürülmeleri daha güç olacaktır.

Bu tez çalışmasında *E. faecalis*'in enfeksiyon ve hayatta kalma mekanizmalarından bir kısmı ele alındı. Buradaki bulguların dentinin ve ilgili diğer konakçı faktörlerinin de dahil edildiği deney düzenlerinde test edilmesinin gerçeğe daha yakın sonuçlar vereceği düşünülmektedir. *E. faecalis*'in enfeksiyon ve hayatta kalma mekanizmaları çok geniş bir konu olup, bu alanda daha fazla araştırmaya ihtiyaç vardır.

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