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Effect of Ca(OH)2 on Survival Ability of a Bacterial Mixture: An In Vitro Study

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Effect of $Ca(OH)_2$ on Survival Ability of a Bacterial Mixture: An *In Vitro* Study

by

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A Thesis submitted in partial satisfaction of
the requirements for the degree of
Master of Science in Endodontics

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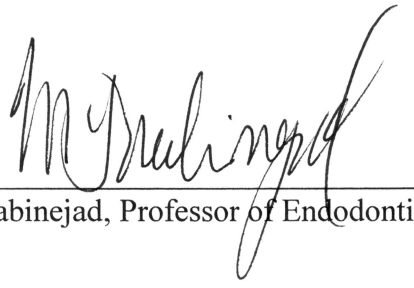

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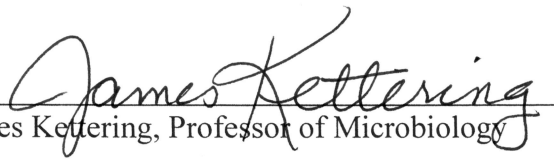

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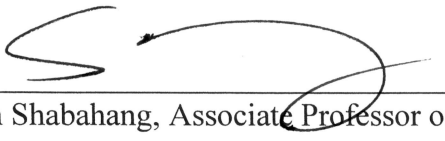

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ABSTRACT

Effect of Calcium Hydroxide on
Survival Ability of a Bacterial Mixture:
An *In Vitro* Study

by

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Loma Linda University, March 2003
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The purpose of this *in vitro* investigation was to evaluate the antibacterial effect of calcium hydroxide on a mixture of nine bacteria associated with endodontic infections at different time intervals and to examine the survival of the same bacteria when in mixture. A total of 102 teeth were used in the first part of the experiment. Eighty teeth were used in eight experimental groups of 10 each, 16 teeth were used for positive and negative controls, and six teeth were used for scanning electron microscopy (SEM). Nine species of bacteria were used in both experiments. These consisted of *Enterococcus faecalis, Lactobacillus bulgaricus, Actinomyces viscosus, Eubacterium alctolyticum, Porphyromonas gingivalis, Fusubacterium nucleatum, Campylobacter rectus, Prevotella buccae,* and *Prevotella denticola.* In the second part of the experiment, the bacterial species were mixed in equal parts in a test tube. The survival ability of each bacterial species was examined at different time intervals. The results of the first experiment showed that after 20 minutes of calcium hydroxide treatment, only *L. bulgaricus,* and *E. faecalis* were recovered. The results further showed that *E. faecalis* was recovered even after a seven day treatment of calcium hydroxide, while *L. bulgaricus* was not recovered at or beyond the 1-day treatment period. The results of the second experiment revealed

that after one day of incubation, *L. bulgaricus* and *E. faecalis* survived in the mixture in the test tube. In addition, the results showed that after four days and up to seven days of incubation, only *L. bulgaricus* survived from the bacterial mixture.

CHAPTER ONE

LITERATURE REVIEW

Endodontic Infections

The aim of non-surgical root canal therapy is the eradication of bacteria from the root canal system (Grossman, 1981, Schilder, 1984) and the sealing of the canal apically (Ingle, Schilder, 1974) and coronally (Ray and Trope, 1995) in order to prevent reinfection of the system.

It has been well established that bacterial leakage into the dental pulp leads to root canal infection. The presence of bacteria in root canals is a prerequisite for the development of pulpal and periradicular infections (Kakehashi *et al*, 1965; Moller, 1981; Fabricius *et al* 1982). Bacteria may enter the pulp through different pathways, including: carious lesions, exposed dentinal tubules (Hoshino *et a.*, 1992), direct pulp exposures, lateral and apical foramina—especially in periodontally involved teeth (Andreans *et al*, 1988), injured cementum (Kiryu, 1990; Love, 1991), and perhaps in certain situations, blood borne bacteria—anachoresis (Robinson and Boling, 1965; Grossman, 1967).

Role of Root Canal Anatomy

Hess (1921) and Davis and coworkers (1972) have shown that root canals contain intricate structures such as isthmi, lateral and accessory canals as well as apical deltas. Because of these complexities, although mechanical cleaning and shaping may greatly reduce the amount of bacteria in the root canal, it is inadequate to eliminate the total bacterial populations in the root canal system (Bystrom and Sundqvist, 1981; Gomes *et al*, 1996; Dalton *et al*, 1998; Siqueira *et al*, 1999). Bystrom and Sundqvist (1981) in an *in vivo* investigation reported a reduction in the number of bacteria in the order of a magnitude of 10^2 to 10^3. In seven out of 17 teeth, bacteria persisted even after five

mechanical treatments. Gomes and colleagues (1996), also in an *in vivo* investigation, tested the susceptibility of different bacteria to biomechanical instrumentation. They concluded from their results that anaerobic bacteria, as well as *Peptostreptococcus* spp. were significantly decreased at the second appointment. Facultative anaerobes, however, were not as susceptible to biomechanical instrumentation. Although 11 out 51 root canals did not have any cultivable bacteria at the second appointment, almost 80% of the canals still had bacteria remaining in the canals. In another *in vivo* study, Dalton and colleagues (1998) compared the effect of nickel titanium rotary instrumentation to stainless steel hand files in measuring bacterial reduction. They found that there was no significant difference between the two techniques in reducing the bacterial count. Although significant reduction was achieved in both groups, there were still cultivable bacteria in 33 out of 46 teeth after three treatments. In an *in vitro* investigation, Siqueira and co-investigators (1999) infected 35 teeth with *E. faecalis* and used three different instrumentation techniques to clean and shape the canals. They reported close to 95% microbial reduction with the Nitiflex® hand, GT® hand, and Profile® .06 rotary files.

Chemical debridement of the root canal system with sodium hypochlorite (NaOCl) significantly increases the efficiency of cleaning and shaping and eradication of bacteria from the root canals (Hand *et al*, 1978; Harrison and Hand, 1981). Ethylene-diaminetetraacetic acid (EDTA) and citric acid, as well as tetracycline have been used in conjunction with NaOCl to remove the smear layer that may be infected with bacteria (McComb and Smith, 1965; Baumgartner *et al*, 1984; Barkhodar, 1993).

Flora of an Infected Root Canal

The flora of infected root canals is mixed in nature. Korzen and associates (1974) infected teeth of 35 gnotobiotic rats with a single bacterial isolate (*Streptococcus mutans)*

and immediately closed the access openings with amalgam. They then accessed and over-instrumented teeth of 35 conventional rats and left them open to the oral environment. They sacrificed the rats at different time intervals and prepared histological sections of the periapical areas of the experimental teeth. They reported that mixed bacterial species present in infected root canals was related to a more severe periapical lesion. Fabricius and co-workers (1982) used 11 bacterial organisms that were isolated from an endodontic infection in monkeys. They mixed the 11 strains in equal proportions, as well as different mixtures of bacteria in combinations and single isolates. They found that mixed infections showed the highest likelihood of inducing radiographically discernable periapical lesions. Pure cultures did not lead to the formation of radiolucent lesions.

Prior to the results reported by Kantz and Henry (1974), the predominant isolates in the earlier studies were facultative streptococci, gram-negative cocci, and lactobacilli. With the introduction of anaerobic isolation techniques, numerous studies have shown the predominating bacteria in endodontic infections are anaerobic in nature (Kantz and Henry, 1974; Baumgartner and Falkler, 1991; Sundqvist *et al*, 1992). Gram-negative black-pigmented *Bacteroides,* which are strict anaerobes, appear to be the predominant species in the mixture. These organisms are currently classified as *Porphyromonas* and *Prevotella* (Shah and Collins, 1988).

Although more than 300 species of bacteria have been identified from root canal infections (Sundqvist, 1976; Sundqvist *et al*, 1989), there are typically five to nine species in an infected root canal. Kantz and Henry (1974) were the first to report the presence of anaerobic bacteria isolated from root canals. Wittgow and Sabiston (1975) sampled 40 teeth that had no response to the electric pulp tester and were scheduled for endodontic treatment. Thirty-six of the teeth had necrotic pulps, and of these, 30

exhibited periapical radiolucencies. Thirty-one of the sampled teeth yielded obligate anaerobic bacteria.

Baumgartner and Falkler (1991) cultured and isolated the bacteria in the apical 5mm of infected root canals. Although they had a small sample size of 10, they were able to observe a common trend. They frequently (68%) isolated strict anaerobes, and invariably (100%) found facultative anaerobes in all examined root canals. The most commonly isolated bacteria were *Actinomyces* species, *Lactobacillus* species, black-pigmented *Bacterioides*, *Peptostreptococcus* species, *Veillonella parvula, Bacterioides buccae, Enterococcus faecalis, Fusobacterium nucleatum,* and *Streptococcus mutans.* Goodman (1977) also found predominantly anaerobic bacteria in infected root canals.

The role of intracanal medicaments in endodontic therapy has been well established (Bystrom *et al*, 1985; Sjogren *et al*, 1991; Oguntebi, 1994). Intracanal medicaments are used to: 1) eliminate remaining bacteria after root canal instrumentation, 2) reduce inflammation of periapical tissues, 3) render canal contents inert and neutralize tissue debris, 4) act as a barrier against leakage from the temporary filling, and 5) help to dry persistently wet canals (Grossman *et al*, 1986; Chong & Pitt-Ford, 1992). Since bacteria have been shown to penetrate the dentinal tubules (Safavi *et al*, 1990; Ørstavik & Haapasalo, 1990; Love, 1996), the intracanal medicament not only should be bactericidal when in contact with the bacteria, but must also be able to penetrate the dentinal tubules in sufficient amounts to eliminate the bacteria present in the tubules.

Calcium Hydroxide as an Intracanal Medicament

Calcium hydroxide ($Ca(OH)_2$) is an extensively researched and the most widely used intracanal dressing. Calcium hydroxide is an alkaline material, which was introduced by Hermann (1920) and has been widely used as an intracanal medicament in

endodontics. It has been widely investigated for its bactericidal activity as an intracanal medicament both *in vivo* (Stevens and Grossman, 1986; Sjogren *et al*, 1991) and *in vitro* (Orstavik & Haapasalo, 1990; Heling *et al*, 1996).

Calcium hydroxide has a pH of 12.5. Due to its alkalinity, most bacteria that are associated with root canal infections are unable to survive (Heithersay, 1975; Bystrom *et al*, 1985). The mechanism by which calcium hydroxide is thought to work is by releasing hydroxyl and calcium ions into the root canal to neutralize the bacteria. In an *in vitro* study, Estrela and associates (1998) found that calcium hydroxide was able to inactivate the bacteria after 12 hours. Calcium hydroxide is said to be bactericidal as well as having tissue-dissolving ability (Filho *et al*, 1999). Its bactericidal ability is believed to be due to its ability to damage the bacterial cytoplasmic membrane. This damage is caused by the free hydroxyl ions that remove hydrogen ions from the phospholipid bilayer membrane (Freeman & Crapo, 1982). Furthermore, the alkaline pH of calcium hydroxide is believed to denature bacterial proteins by breaking the ionic bonds of the tertiary structure of proteins (Voet & Voet, 1995). Finally, calcium hydroxide is believed to be bactericidal by having the hydroxyl ions splitting the bacterial DNA strands leading to the destruction of genetic material (Imlay and Linn, 1988).

Calcium hydroxide powder has been used in conjunction with sterile water, saline, glycerin, as well as camphorated paramonochlorophenol (CPMC) (Rivera & Williams, 1994; Alçam *et al*, 1998, Çalişkan *et al*, 1998; Siqueira & de Uzeda, 1998). The type of vehicle used with calcium hydroxide determines the rate of disassociation of calcium hydroxide that releases the calcium and hydroxide ions. Water and saline help release the ions of calcium hydroxide at a rapid rate, whereas glycerin or camphor solutions provide a slower dissociation of the calcium and hydroxide ions. With respect

to the delivery of calcium hydroxide into the canals, no difference has been found with the various carriers.

Calcium hydroxide paste has been shown to eradicate the bacteria from root canals in one week (Sjogren *et al*, 1991). In an *in vivo* investigation involving teeth with radiographic lesions, Sjogren and coworkers have shown that a 7-day treatment with calcium hydroxide eradicates the bacteria from the root canal system. However, in the same study, a 10-minute application of $Ca(OH)_2$ paste, of equal concentration, was not able to consistently eliminate the bacteria from the teeth.

Bacteria have competitive and synergistic associations with other microorganisms (Simonson *et al*, 1992; Sundqvist *et al*, 1992). Gibbons and McDonald (1980) showed that hemin and vitamin K are essential elements for the growth of certain strains of *Bacteroides melaninogenicus*. Socransky and Gibbons (1965) have shown that some anaerobic bacteria such as the *Porphyromonas* and *Prevotella* spp. may acquire these nutritional needs from gram-positive bacteria.

Bacteria in the Dentinal Tubules

Numerous studies have used anaerobic and facultative bacteria to infect dentinal tubules. Some of these studies have also determined the distance that various bacteria penetrate into the dentinal tubules (Akpata and Blechman, 1982; Ørstavik and Haapasalo, 1987, 1990; Love, 1996; Berkiten *et al*, 2000). In an *in vitro* study, Akpata and Blechman (1982) infected dentinal tubules of human teeth with *Streptococcus sanguis**. They showed that *S. sanguis* requires 14 days to penetrate the dentinal tubules. Ørstavik and Haapasalo (1987) infected freshly extracted bovine teeth with *E. faecalis**, and showed that this species penetrates the dentinal tubules 300μm to 1mm. In 1990, the same investigators showed that it takes *E. faecalis* only two days to penetrate the entire depth

of the circumpulpal dentin. They confirmed that *S. sanguis* required 14 days to penetrate the depth of the dentin. *E. coli**, on the other hand, is only able to penetrate 600μm even after 14 days of incubation. An interesting finding by Haapasalo and Ørstavik (1990) was that *E. faecalis* is able to survive for 10 days, even after removing the nutritional support. In a histological investigation, Love (1996) demonstrated that *Streptococcus gordonii** penetrates the dentinal tubules to a maximum distance of 200μm in the cervical and midroot section of the tooth, and to a distance of 60μm in the apical segment of the root. Berkiten and coworkers (2000) showed that *S. sanguis* penetrates the dentinal tubules to a range of 50-850μm, with a mean penetration of 382μm. They also showed *Prevotella intermedia** has a penetration range of 0-275μm.

To test the effects of intracanal medicaments, investigators have infected dentinal tubules with various bacterial species. Haapasalo and Ørstavik (1987) infected bovine dentinal tubules with *E. faecalis.* They found that calcium hydroxide is unable to kill *E. faecalis* even in the superficial portion of the tubules. Stuart and coworkers (1991) inoculated teeth with a mixture of *Streptococcus mutans**, *Actinomycosis viscosus**, and *P. gingivalis** or *Bacteroides fragilis** to test the effect of calcium hydroxide on these organisms . They reported that Pulpdent® had a significantly higher antibacterial activity than CMCP and formocreosol on *S. mutans* and *P. gingivalis* or *B. fragilis*. They found that there was no difference in the antibacterial activity against *A. viscosus.*

Siqueira and de Uzeda (1996) infected dentinal tubules with a facultative anaerobe (*E. faecalis*), and two obligate anaerobic bacteria (*A. israelii** and *Fusobacterium nucleatum**). They then tested the disinfecting ability of calcium hydroxide on the dentinal tubules, and found that calcium hydroxide mixed with sterile saline is ineffective against *E. faecalis* and *F. nucleatum* even after 1 week of exposure.

However, when mixed with CMCP, calcium hydroxide was able to completely disinfect the tubules after 1 day of exposure.

In previous studies, dentinal tubules were either infected with a single isolate (Ørstavik and Haapasalo, 1987; Siqueira and de Uzeda, 1996; Perez *et al*, 1996; Berkiten *et al*, 2000) or with two or three different bacterial species in combination (Stuart *et al*, 1991; Barrieshi *et al*, 1997; Estrela *et al*, 1998). When the bacteria were mixed, the interaction—i.e. synergistic and competitive effect of the bacteria on each other, had not been considered. Therefore, the results of the test medicament may have not been solely due to the effect of the medicament.

The purpose of this investigation was to study the effect of calcium hydroxide on a combination of nine different bacterial species commonly found in infected root canals over eight time intervals and to determine the survival ability of these organisms when mixed in a test tube in different combinations at various time intervals.

*See Table 1-1 for relative bacterial sizes.

Table 1-1— Properties of bacteria used to infect dentinal tubules

Bacterial Species	**Gram Stain**	**Morphology**	**Size in µm x µm**	**Oxygen Requirement**
A. israelii	gram +	Filamentous Rods	0.2-1 X 1.5-5	Anaerobic
**A. viscosus*	gram +	Rods w/ Branching	0.2-1 X 1.5-5	Anaerobic
**C. rectus*	gram -	Rod	0.2-0.5 X 0.5-5	Anaerobic
**E. alactolyticum*	gram +	Bacillus	0.3-0.6 X 1.6-7.5	Anaerobic
E. coli	gram -	Motile Rod	0.5 X 3	Facultative Anaerobe
**E. faecalis*	gram +	Cocci	0.5-1 in diameter	Facultative Anaerobe
**F. nucleatum*	gram -	Fusiform	0.4-0.7 X 3-10	Anaerobic
**L. bulgaricus*	gram +	Short rods in chains	0.5-0.8 X 2-9	Facultative Anaerobe
**P. buccae*	gram -	Rod	0.5-0.7 X 0.8-8	Anaerobic
**P. denticola*	gram -	Rod	0.5-0.7 X 0.7-6	Anaerobic
**P. gingivalis*	gram -	Bacillus	0.5-0.8 X 1.5-4.5	Anaerobic
P. intermedia	gram -	Rod	0.4-0.7 X 1.5-2	Anaerobic
S. gordonii	gram +	Cocci	1.2-1.8 in diameter	Facultative Anaerobe
S. mutans	gram +	Cocci	1-2 in diameter	Facultative Anaerobe
S. sanguis	gram +	Cocci	0.8-1.2 in diameter	Facultative Anaerobe

*Bacteria Used in Our Mixture

CHAPTER TWO

ANTIBACTERIAL EFFECT OF CALCIUM HYDROXIDE ON A MIXTURE OF BACTERIA: AN *IN VITRO* INVESTIGATION

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Abstract

The purpose of this *in vitro* investigation was to determine the antibacterial effect of calcium hydroxide on a mixture of nine bacteria associated with root canal infections at various time intervals. The root canals of 102 extracted human teeth were cleaned and shaped in this investigation. After sterilization and removal of the smear layer, the root canals of 94 samples were contaminated with a mixture of nine known bacterial species. The test bacteria included: *Enterococcus faecalis, Lactobacillus bulgaricus, Actinomyces viscosus, Eubacterium alactolyticum, Porphyromonas gingivalis, Fusobacterium nucleatum, Campylobacter rectus, Prevotella buccae,* and *Prevotella denticola.* Eighty root canals were medicated with calcium hydroxide [$Ca(OH)_2$] in eight different time intervals (10 in each group). Root canals of eight teeth were not medicated and were used as positive controls. The root canals of eight samples were not contaminated and served as negative controls. The remaining six contaminated teeth were used for SEM examination. The results showed that after 20 minutes of calcium hydroxide treatment, *L. bulgaricus* and *E. faecalis* were the only bacteria, which survived. Live *E. faecalis* were recovered from root canals of the majority of teeth, even after a seven-day treatment with $Ca(OH)_2$.

Introduction

The aim of non-surgical root canal therapy is the eradication of bacteria from infected root canals and sealing them apically and coronally in order to prevent their reinfection.[1] The presence of bacteria in root canals is a prerequisite for the development of pulpal and periradicular infections.[2] Bacteria may enter the pulp through carious lesions, exposed dentinal tubules, lateral and apical foramina—especially in periodontally involved teeth, injured cementum, and perhaps in certain instances from blood-borne bacteria—anachoresis.[3]

The flora of infected root canals is mixed in nature and, although more than 300 species of bacteria have been identified from infected root canals,[4] there are typically five to nine predominant species in an infected root canal.[5] Flora of infected root canals contain mainly gram-negative black-pigmented anaerobic *Bacteroides* spp.[5] These organisms are currently classified as *Porphyromonas* and *Prevotella.*

Studies have shown that root canals contain intricate structures such as isthmi, lateral and accessory canals as well as apical deltas.[6] Because of these complexities, mechanical cleaning and shaping alone is inadequate to eliminate, completely, bacterial populations from the root canal system. Chemical debridement of the root canal system with sodium hypochlorite (NaOCl) significantly increases the efficiency of cleaning and eradication of bacteria from the root canals.[7] Chelating agents such as ethylene-diaminetetraacetic acid (EDTA), as well as citric acid, and doxycycline have been used in conjunction with NaOCl to remove the smear layer that may be infected with bacteria.[8,9] Byström and Sundqvist have shown that even after careful chemo-mechanical cleansing, root canals remain infected.[10]

Intracanal medicaments are used to: 1) eliminate remaining bacteria after root canal instrumentation, 2) reduce inflammation of radicular tissues, 3) render canal contents inert and neutralize tissue debris, 4) act as a barrier against leakage from the temporary filling, and 5) help to dry persistently wet canals. [11] Since bacteria have been shown to penetrate the dentinal tubules,[12] the intracanal medicament not only should be bactericidal when in contact with the bacteria, but must also be able to penetrate the dentinal tubules in sufficient amounts to eliminate the bacteria present in the tubules.

Calcium hydroxide [$Ca(OH)_2$] is an alkaline material, and it is widely used as an intracanal medicament in endodontics.[13] It has been investigated for its bactericidal activity as an intracanal medicament both *in vivo* [14] and *in vitro*. [12,15-18] $Ca(OH)_2$ has a pH of 12.5. Most bacteria that are associated with root canal infections are unable to survive this level of alkalinity. In an *in vitro* study, Estrela and coworkers[15] found that when $Ca(OH)_2$ is used on a combination of six bacterial species, it is able to inactivate all six species after 12 hours.

A review of the literature revealed an absence of data demonstrating the ability of $Ca(OH)_2$ to sterilize root canals in less than seven days. To determine the amount of time necessary for $Ca(OH)_2$ to exert its bactericidal efficacy, Sjogren and associates[14] used this material for 10 minutes and 7 days in teeth with necrotic pulps and radicular radiolucencies. They found that a 10-minute treatment is not sufficient to kill all bacteria, however, 7 days of $Ca(OH)_2$ application is able to render the canals sterile. The knowledge that $Ca(OH)_2$ is able to render an infected root canal sterile in a shorter period of time would be useful to the clinician in certain clinical situations. The purpose of this investigation was to test the antibacterial effect of $Ca(OH)_2$ on a combination of nine

different bacterial species commonly found in infected root canals at various time intervals.

Material and Methods

A total of 102 extracted single rooted human teeth were used in this study. Each tooth had a total length of 21-25 mm. The teeth were divided into eight experimental groups of 10 teeth in each group. Six teeth were used for SEM analysis after initial smear layer removal, bacterial contamination, and recreation of the smear layer. The remaining 16 teeth were used as positive and negative controls.

Treatment—Tooth Preparation

The surfaces of extracted teeth were cleaned using hand scalers. Access to the root canal of each tooth was prepared through the lingual or occlusal surface. An initial K-type file (size 10) was used to determine the working length by penetrating the apical foramen and pulling back to the visible clinical foramen. A 35/.12 Greater Taper Rotary (GTR®--Tulsa Dental, Oklahoma, USA) file was used to achieve a pre-flare in the coronal aspect of the root canal. GTR® files 20/.12, .10, .08, .06 were used serially in a crown down technique to shape the canals.

Five milliliters (ml) of 5.25% NaOCl was used for irrigation during cleaning and shaping. After complete chemo-mechanical debridement of the canals, each canal was irrigated with 2ml of 17% EDTA for 4 minutes in order to remove the smear layer. After flushing with 3ml 5.25% NaOCl, a 5% sodium thiosulfate solution was used to inactivate any remaining NaOCl as previously recommended. [14]

Following sterilization in a steam autoclave at 121°C at 15 PSI for 20 minutes, the teeth were sealed at the apices with sticky wax under aseptic conditions and stored in sterile humid containers. Two teeth were chosen at random prior to bacterial infection to ensure patency of the dentinal tubules using a scanning electron microscope (SEM). Furthermore, two teeth from the experimental group after smear layer removal, bacterial contamination, as well as two teeth after the recreation of the smear layer were split and examined for bacterial penetration under SEM.

SEM Preparation

After splitting each tooth with a disk, the halves were placed in a gluteraldehyde solution for 24 hrs, rinsed twice with a sodium buffered saline (pH 7.2), treated with osmium tetra-oxide for one hour, rinsed in ascending concentrations of ethyl alcohol (30%-100%), and then placed in a dessicator for 24 hrs. The samples were then mounted on special buttons and coated with 25μm of gold-palladium (Au-Pd). The samples were examined at a magnification of 500X to 10,000X at random in the coronal, mid-root, and apical areas of the root canals. The samples were checked for dentinal tubule patency, bacterial infection, and presence of the smear layer depending on the purpose of the examination.

Bacterial Sample Preparation

Nine different bacterial strains—*Campylobacter rectus* (ATCC 33238), *Enterococcus faecalis* (ATCC 4082), *Eubacterium alactolyticum* (ATCC 23263), *Actinomyces viscosus* (ATCC 19246), *Fusobacterium nucleatum* (ATCC 49256), *Lactobacillus bulgaricus* (ATCC 7517), *Porphyromona gingivalis* (W83), *Prevotella*

buccae (ATCC 33689), and *Prevotella denticola* (ATCC 35308)—were used in this experiment. All but two of the bacteria were obtained from ATCC. *E. faecalis* and *P. gingivalis* were used from frozen stocks from Loma Linda University Department of Microbiology (LLUM). The selected bacteria for this experiment were strains that are commonly found in teeth with necrotic pulps and periradicular radiolucencies.[14] The bacteria were anaerobically cultured (37°C) in pre-reduced anaerobically sterilized (PRAS) broth (Anaerobic Systems, Morgan Hill, California) supplemented with vitamin K and heme in an anaerobic chamber (Coy Laboratories, Ann Arbor, Michigan) for two weeks.

Bacterial Mixture

Using optical density measurements (Beckman DU-650 spectrophotometer (Fullerton, CA)), a mixture of the bacterial strains was obtained containing approximately 1×10^7 bacteria from each of the nine strains. Calculations were made based on the fact that approximately 4.3×10^8 bacteria have an absorbance of 1.0 at 640nm. Based on the recorded absorbance of each bacterial sample, appropriate amounts were mixed to a total of 1×10^7 bacteria from each strain (Table 1). From this mixture, 1×10^7 ($1/9^{th}$ of the total bacterial mixture) bacteria were placed in PRAS (Anaerobic Systems) broth and 20µl was introduced into each experimental and positive control tooth. The teeth were visually inspected every 24 hours with respect to the nutrient level and were replenished with up to 20µl of fresh sterile PRAS (Anaerobic Systems) broth to maintain bacterial viability. The teeth were stored in an anaerobic chamber (Coy Laboratories) with an atmospheric pressure of 10% hydrogen and 5% carbon dioxide in nitrogen at 37°C.

After contamination for one week, smear layer was recreated using rotary instruments (#3 Peeso Reamer—Union Broach, Union city, NJ) to entomb the bacteria in the dentinal tubules. The presence of bacteria in the dentinal tubules was confirmed by obtaining dentinal shavings from the experimental teeth using a Peeso Reamer (Union Broach) and placing the shavings in liquid media and culturing for bacterial growth. After confirmation of the presence of bacteria inside the dentinal tubules, a randomly selected tooth was split and prepared for SEM analysis to confirm the presence of smear layer on the dentin surface.

$Ca(OH)_2$ paste, Ultracal® (Ultradent, South Jordan, UT), was placed in the root canal of each experimental tooth. The canals were treated with $Ca(OH)_2$ paste for 20 minutes, 1 day, 2, 3, 4, 5, 6 and 7 days. The access cavities were sealed with Gamma-radiated Cavit®. All procedures were performed in an anaerobic chamber at 37°C.

At the end of each experimental time, the bulk of the $Ca(OH)_2$ was removed using a sterile spoon excavator and sterile paper points. The $Ca(OH)_2$ and the paper points were placed on Brucella anaerobic agar plates (Anaerobic systems , Morgan Hill, California) and cultured to determine presence of bacteria. This was done to ensure no viable bacteria were removed with this procedure. After irrigation with sterile water, and drying with sterile paper points, the canals were sampled using sterile paper points saturated with sterile water. All procedures were performed in an anaerobic chamber (Coy Laboratories, Ann Arbor, Michigan). The paper points were then placed in PRAS broth (Anaerobic Systems). "Samples that demonstrated turbidity were streaked on brucella anaerobic agar plates (Anerobic Systems)" to identify the isolated colonies. These organisms were identified as follows: They were initially Gram stained, and based on the results of the Gram staining, they were subjected to catalase and oxidase tests,

antibiotic susceptibility tests and a bacterial identification system (IDS) (Bio-meriuex, Marcy Petoile, France) (Figure 1).

Bacterial species as well as the relative percentage of bacteria recovered from the experimental teeth were measured. Fresh PRAS broth (Anaerobic Systems) was added to recover any potentially remaining bacteria in the root canals that yielded negative cultures. The root canals of these teeth were sampled again weekly for a period of five weeks to ensure that no bacteria were present. If bacteria were recovered, the relative percentage of various strains that were recovered was calculated.

A positive and negative control tooth for each experimental group was used. Eight root canals (one for each experimental time) that were inoculated with the bacterial mixture, and had no $Ca(OH)_2$ treatment were used as positive controls. The canals of these teeth were treated with sterile broth. Positive control samples were cultured for bacteria at the same experimental times as from the experimental groups to ensure that the bacterial mixture still contained viable organisms. Eight root canals (one for each experimental time) that were filled with sterile broth and had no bacterial infection served as the negative control samples. The negative control specimens were also cultured to determine bacterial growth at the different experimental times to ensure that the experimental model was valid, and that no contamination had occurred during the experimental procedures.

Bacterial Recovery and Identification

Since the initial bacterial strains were known, contamination with other bacterial strains during the microbiological procedures would have been easily recognized. In order to identify recoverable bacteria, paper points were used to sample bacteria from

each canal and placed in sterile broth. Furthermore, dentin shavings collected using Peeso reamers #4 (Union Broach) were placed in sterile broth. After one week, the samples were plated on Brucella anaerobic plates (Anaerobic Systems) and grown in the anaerobic chamber (Coy Laobratories). If bacteria were recovered from the root canal system, the species were identified using Gram staining, antibiotic sensitivity tests, and other biochemical tests (Figure 1). Contamination of the bacteria by other strains was ruled out using the criteria outlined in Figure 1. The presence or absence, as well as an estimate of the relative percentage from each bacterial species that survived after each experimental time were calculated and analyzed using a two-sample test for binomial proportions.

Results

Figures 2-4 show the SEM observations after EDTA treatment, contamination of the root canals, and recreation of the smear layer, respectively.

The samples in the negative control group showed no bacterial growth at all time periods. The positive control teeth, however, showed only *L. bulgaricus* and *E. faecalis* at each time period sampled. *L. bulgaricus* was recovered from the root canal (paper points) while *E. faecalis* was recovered from the dentinal tubules (dentin shavings). The remaining seven bacterial species were not recovered.

In the experimental groups, results showed that after 20 minutes of calcium hydroxide treatment, *L. bulgaricus* and *E. faecalis* survived the $Ca(OH)_2$ treatment. *E. faecalis* was recovered from 8 out of 10 teeth. *L. bulgaricus* was recovered from 9 out of 10 canals. The difference in the bactericidal effect of calcium hydroxide on *L. burgaricus* and *E. faecalis* was not statistically significant ($p > 0.05$). As in the positive control

samples, none of the other seven bacterial species were recovered from the root canals of any of the teeth. After one day, and up to seven days of $Ca(OH)_2$ treatment, *E. faecalis* was the only bacterial species that was still able to survive in the majority of the samples. No *L. bulgaricus* was recovered beyond the 20-minute test period (Table 3). However, there was a statistically significant reduction of *E. faecalis* and *L. bulgaricus* at all examined calcium hydroxide treatment times (at T_{20min}, P= .0358; at $T_{1\text{-}5days}$, P=.0358; at T_{6days}, P= .0078; and at T_{7days}, P=.0016). Calcium hydroxide was able to eradicate *L. bulgaricus* in all samples in less than one day of treatment. However, calcium hydroxide was not able to consistently eliminate *E. faecalis* even after a 7-day treatment in the majority of infected root canals.

Discussion

In previous investigations, $Ca(OH)_2$ has been either used *in vitro* against single bacterial species[12, 16] or against two to three bacterial species in combination.[15] One *in vivo* investigation exists where the effect of $Ca(OH)_2$ was tested against polymicrobial flora of infected teeth. [14]

Our results showed that when bacterial organisms (used in this experiment) are mixed *in vitro* only *E. faecalis* and *L. bulgaricus* survived in the mixture. In addition, we showed that $Ca(OH)_2$ was unable to consistently eliminate *E. faecalis* even after seven days of treatment.

When bacteria are used to infect root canals and dentinal tubules *in vitro*, the teeth should be sterilized in order to prevent contamination and show accurate results. Stevens and Grossman have shown that sterilization of teeth in a steam autoclave does not affect the ability of bacteria to penetrate the dentinal tubules.[13] Pashley and coworkers[18]

sterilized extracted human teeth in a steam sterilizer and investigated the permeability of the dentin. They compared their results with the permeability of dentin in teeth that were not sterilized (controls), with the permeability of dentin sterilized in ethylene oxide. Their results show that there is no difference in the permeability of the dentin among the three groups.

Stuart and colleagues[19] mixed *S. mutans, A. viscosus,* and *B. gingivalis,* or *B. fragilis* and tested the effect of Pulpdent®, a slurry of sterile water and $Ca(OH)_2$, formocreosol, and CMCP on these mixtures. Their findings show a significant reduction in the bacterial count for all tested medications compared to the untreated controls. The reported 99.9% reduction of *B. gingivalis* corresponds to 1.61-100 x 10^3 bacteria remaining from the initial count of 1.61 – 100 x 10^6. Small numbers of bacteria would rapidly multiply to repopulate the teeth in a short time. Finally, the presence or absence of bacteria was not confirmed in the dentinal tubules. Therefore, bacteria may have infected the tubules, and not have been eliminated by the effect of $Ca(OH)_2$.

Estrela *et al*[15] tested the effect of $Ca(OH)_2$ for periods of 0, 1, 2, 6, 12, 24, 48, 72 hours and 7 days. The bacterial species tested in their investigation were aerobic gram-positive cocci (*M. luteus, S. aureus,* and *Streptococcus sp.*), aerobic gram-negative coccus (*E. coli,* and *P. aeruginosa*), and anaerobic gram-negative rods (*F. nucleatum*). Their results showed that $Ca(OH)_2$ is effective in eliminating *M. luteus, F. nucleatum* after 12 hours, *Streptococcus* spp. after 24 hours, *E. coli* after 48 hours, and *S. aureus* and *P. aeruginosa* after 72 hours. In their study[15], Estrela and coworkers made no attempt to determine the survival ability of the bacteria in the absence of $Ca(OH)_2$. Therefore the elimination of the bacteria cannot be solely attributed to the use of $Ca(OH)_2$.

Although our observations include similar results as the previous two investigations, [15,19] we have taken the experiment a step further by considering bacterial interaction. In doing so, we have come to different conclusions from the previous reports. The results of our investigation are in agreement with those of a number of previous investigations suggesting that $Ca(OH)_2$ is ineffective against *E. faecalis.* [12,13,15-17]

Sjogren and colleagues[14] showed that $Ca(OH)_2$ is not effective in eliminating the bacteria from root canals in a 10-minute period, but a 7-day treatment is effective. Our results agree with the results of Sjogren and his coworkers if the presence of *E. faecalis* is not examined in the dentinal tubules. A possible reason for the apparent conflict between Sjogren's study[14] and our data is that Sjogren and coworkers[14] did not use any methodology to see if any viable bacteria exist in the dentinal tubules. Because they had the teeth sealed for a period of up to five weeks, they may have assumed that if bacteria were present in the tubules, a positive culture would have been obtained. However, since the smear layer was not removed, bacteria may have been entombed in the tubules where $Ca(OH)_2$ has a lesser chance of reaching the bacteria and the bacteria would not find their way back to the main canal for culturing.

Although the samples in our study were infected with nine different bacterial species, only *E. faecalis* and *L. bulgaricus* survived. Therefore, the effect of $Ca(OH)_2$ was actually being tested against only two bacterial species. The remaining seven species did not survive in the mixture by the end of day four of the one-week infection time. The fate of these strains, and the mechanisms by which they were eliminated was investigated. The results of that study showed that when a bacterial mixture containing *L. bulgaricus* was inoculated with six other bacterial species (including *E. faecalis),* only *L. bulgaricus* was able to survive after four days in the mixture.[20] The results of the

$Ca(OH)_2$ treatment during the various time periods showed that *L. bulgaricus* was eliminated, but that *E. faecalis* was not. These results are in agreement with those reported by Molander *et al.*[17]

Since in our experiment the smear layer was recreated to entomb the bacteria in the dentinal tubules, it follows that *E. faecalis,* which had penetrated the dentinal tubules would be entombed there. Furthermore, since there were no bacteria recovered from the root canal using the paper points, it appears that $Ca(OH)_2$ eliminated the *L. bulgaricus* from the root canal. However, since, even after seven days, *E. faecalis* was recovered from the dentinal tubules, through dentinal shavings, either $Ca(OH)_2$ was not able to penetrate into the tubules to eradicate the *E. faecalis*, or it was ineffective against *E. faecalis.*

Our results also showed that when certain bacterial species are mixed, bacterial interaction, and competition takes place favoring survival of certain species over others. In our investigation, only *L. bulgaricus* and *E. faecalis* were able to survive in a mixture of nine bacterial species. Based on our results, it appears that the use of $Ca(OH)_2$ is not effective in eliminating *E. faecalis.* A new method or material is needed to eradicate bacteria such as *E. faecalis* from infected root canals to improve treatment outcome.

Acknowledgements

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Figure Legends

Figure 1. Identification of bacteria according to the procedural chart

Abbreviations:

GPC—Gram-positive cocci

GPB—Gram-positive bacilli

GNB—Gram-negative bacilli

CHOC—Chocolate agar

PYR-pos—Pyruvate positive

IDS—Identification system

POS—Positive

NEG—Negative

Figure 2. SEM photomicrograph demonstrating open tubules after removal of the smear layer and prior to bacterial contamination. Original magnification X 3000.

Figure 3. SEM photomicrograph demonstrating presence of Bacteria in the Contaminated Root Canal. Original Magnification X 5000.

Figure 4. SEM photomicrograph demonstrating the smear layer after bacterial contamination at 500X magnification.

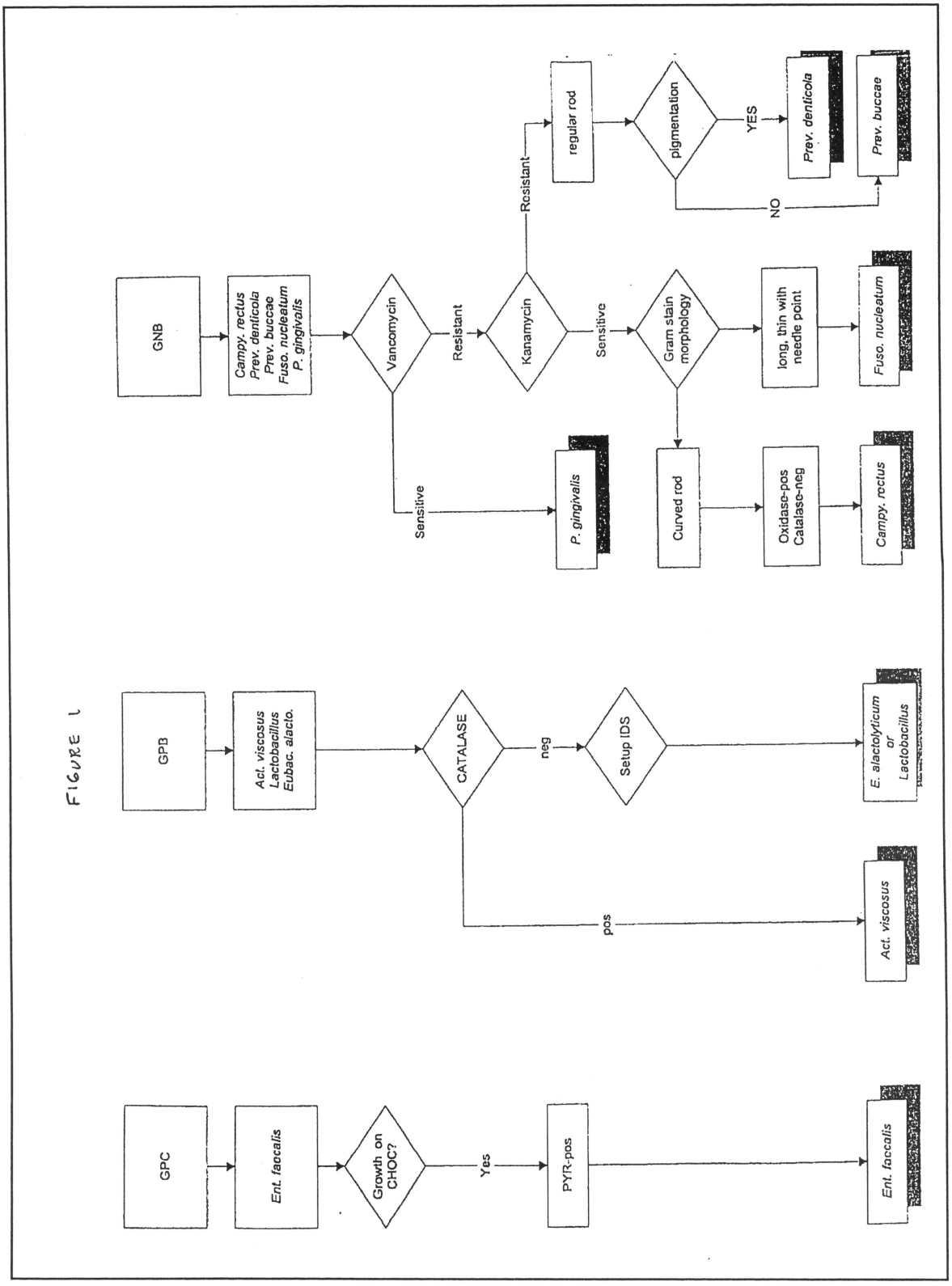
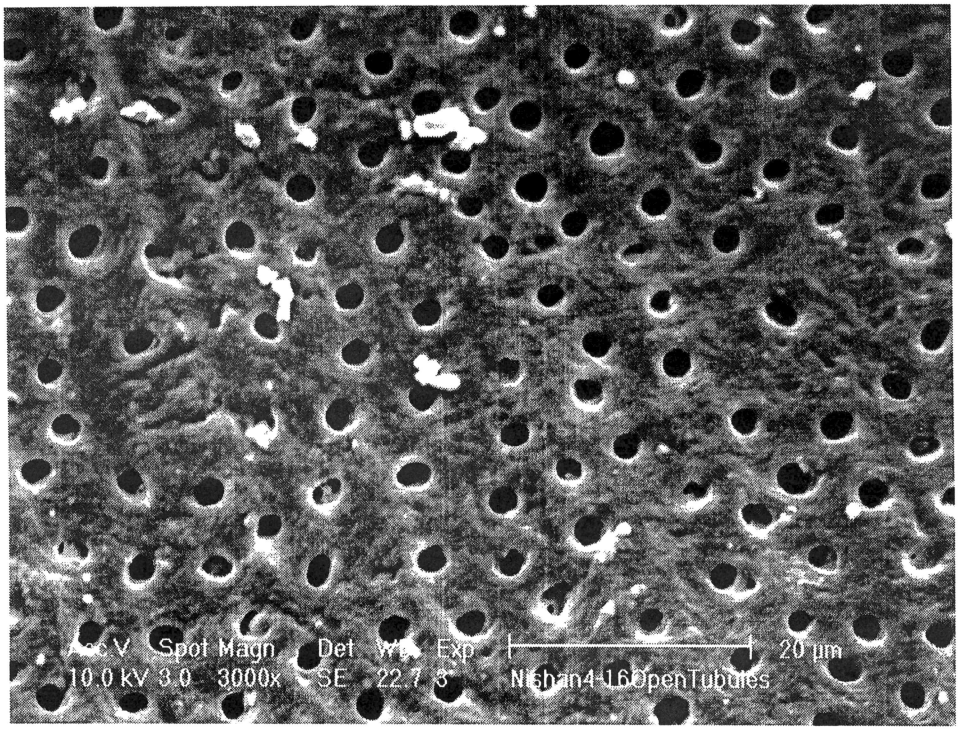


Figure 2-1—Identification of bacteria according to the procedural chart.


Acc.V Spot Magn Det WD Exp
10.0 kV 3.0 3000x SE 22.7 3
20 µm
Nishan4-16OpenTubules

CHAPTER THREE

SURVIVAL ABILITY OF A MIXTURE OF BACTERIA: AN *IN VITRO* INVESTIGATION

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Running title: "Effect of calcium hydroxide on a Mixture of Bacteria"

Abstract

The purpose of this *in vitro* investigation was to test the survival ability of various mixtures of seven bacterial species in a test tube cultured over various time intervals. Seven bacterial species consisting of *Enterococcus faecalis, Lactobacillus bulgaricus, Actinomyces viscosus, Eubacterium alctolyticum, Porphyromonas gingivalis, Fusubacterium nucleatum,* and *Prevotella buccae* were used in this experiment as a mixture in a test tube under anaerobic conditions for a period of 1, 2, 3, and 7 days. The results of the experiment revealed that after one day, and up to three days of mixture, only *L. bulgaricus*, and *E. faecalis* survived in the test tube. After the fourth day, and up to seven days, only *L. bulgaricus* survived in the test tube. Based on our results, it appears that *L. bulgaricus* was the only bacterial species that was able to survive in an *in vitro* mixture after four days.

Introduction

The flora of infected root canals is polymicrobial in nature.[1-4] Prior to the results reported by Kantz and Henry, [2] the predominant isolates reported in the literature were facultative streptococci, gram-negative cocci, and lactobacilli. With the introduction of anaerobic isolation techniques, numerous investigations have shown that the predominating bacteria in infected root canals are anaerobic microorganisms.[1-4] Gram-negative black-pigmented *Bacteroides;* which are strict anaerobes, seem to predominate. These organisms are currently classified as *Porphyromonas* and *Prevotellas.* [5]

Although more than 300 species of bacteria have been identified from infected root canals,[4] there are typically five to nine predominant species in an infected root canal.[2,3] The most commonly isolated bacteria from infected root canals are *Actinomyces* species, *Lactobacillus* species, black-pigmented *Bacterioides*, *Peptostreptococcus* species, *Veillonella parvula, Bacterioides buccae, Enterococcus faecalis, Fusobacterium nucleatum,* and *Streptococcus mutans.* [2-4]

Bacterial interaction in the form of competitive or synergistic associations is well documented.[6-8] Socransky and Gibbons[8] showed that hemin and vitamin K are the essential elements for the growth of certain strains of *P. melaninogenica*. They showed that some anaerobic bacteria such as the *Porphyromonas* and *Prevotella* acquire these nutritional needs from gram-positive bacteria.

Stuart *et al* inoculated teeth with a mixture of *Streptococcus mutans, Actinomycosis viscosus,* and *Bacteroides gingivalis* or *Bacteroides fragilis* to test the effect of calcium hydroxide [$Ca(OH)_2$] on these organisms.[9] They reported a 99.9% reduction of *B. gingivalis* after one hour of treatment with this material. Estrela and associates used *Micrococcus luteus, Staphylococcus aureus, Fusobacterium neculeatum,*

Pseudomonas aeruginosa, Escherichia coli, and *Streptococcus* spp. to make four bacterial mixtures.[10] They tested the effect of $Ca(OH)_2$ for time periods ranging from 1h to 7 days on aerobic gram-positive cocci (*M. luteus, S. aureus,* and *Streptococcus sp.*), aerobic gram-negative cocci (*E. coli,* and *P. aeruginosa*), and an anaerobic gram-negative rod (*F. nucleatum*). Their results showed that $Ca(OH)_2$ is effective in eliminating *M. luteus,* and *F. nucleatum* after 12 hours, *Streptococcus sp.* after 24 hours, *E. coli* after 48 hours, and *S. aureus* and *P. aeruginosa* after 72 hours. These investigators did not culture the dentinal tubules to determine the presence or absence of bacteria. In addition, they did not use controls to evaluate the potential for interaction of the bacteria in the absence of $Ca(OH)_2$.

These studies[9,10], did not consider the synergistic or competitive effect of the bacteria with one another. Therefore, the results that were attributed to the test medicament may have been influenced by the interaction of the bacteria and not solely due to the effect of the medicament.

In a previous investigation, we evaluated the effect of $Ca(OH)_2$ on a mixture of nine different bacterial species for various time intervals.[11] Our results showed that after 20 min. of incubation of bacteria, only two out of nine bacterial species (*Enterococcus faecalis,* and *Lactobacillus bulgaricus*) survive. The purpose of this investigation was to determine the ability of seven bacterial species to survive in various combination in a test tube for various time intervals.

Material and Methods

Bacterial Preparation

Seven bacterial strains—*Enterococcus faecalis* (ATCC 4082), *Eubacterium alactolyticum* (ATCC 23263), *Actinomyces viscosus* (ATCC 19246), *Fusobacterium nucleatum* (ATCC 49256), *Lactobacillus bulgaricus* (ATCC 7517), *Porphyromona gingivalis* (W83), *Prevotella buccae* (ATCC 33689)--were cultured in prereduced anaerobically sterilized (PRAS) broth (Anaerobic Systems, Morgan Hill, California) for two weeks. Viability and purity were confirmed, and Gram staining was performed to ensure absence of contamination.

Bacterial Mixture

Bacterial concentration was ruled out according to the method previously described.[12] The bacterial mixture in the PRAS broth (Anaerobic Systems, Morgan Hill, CA) containing thioglycolate was stored in an anaerobic incubator (Coy Laboratories, Ann Arbor, Michigan) at 37°C for the duration of the experiment.

Although the bacterial viability and purity were being monitored prior to the mixture of the bacteria, each bacterial strain had to be re-isolated after mixture with other bacterial species. Furthermore, certain control mixtures were utilized in the experiment in order to determine the accuracy of the experimental procedures, as well as the reliability of the results. A total of 16 different combinations of the bacteria were mixed. Table 1 shows the different combinations of the bacterial species. Mixture I contained the three anaerobic Gram-negative bacilli. Mixture II contained the three anaerobic Gram-negative bacilli as well as the anaerobic Gram-positive cocci (*E. alactolyticum)*. Mixture III had the anaerobic bacteria as well as *L. bulgaricus*. Mixture IV contained the anaerobic

bacteria as well as the facultative anaerobe *E. faecalis.* Mixture VI contained the two gram-positive facultative organisms *E. faecalis,* and *L.* Mixtures VII through XVI contained each bacterial species with either *E. faecalis* or *L. bulgaricus* .

The bacterial mixture from each test tube was diluted 100X and plated on Brucella anaerobic agar plates (Anaerobic Systems) (figure 1). The procedures for culturing and identification of the bacteria were similar to those reported in our previous investigation. [11] In addition, it is important to note, that while the seven bacterial species were mixed and cultured in anaerobic conditions, they were also grown concomitantly separately. Individual bacterial species were tested for viability at the same time periods as the mixture to ensure that individual species were still alive. All seven bacterial species were viable at all tested periods when grown individually. This procedure allowed us to surmise that bacterial interaction was the cause for *L. burlgaricus* to remain as the only viable species in our mixture.

Bacterial recovery and isolation times were performed immediately, 24h, 72h and 7 days after mixture according to the procedures described previously.[11] The data was analyzed using two sample test for binomial proportions.

Results

All bacteria included in the mixture were re-isolated immediately after mixture. However, the recovered isolates showed a relative change from the initial percentages at time T_0 (pre-mixture) (Table 2). At 24 hours, only *E. faecalis* and *L. bulgaricus* were recovered. *L. bulgaricus* accounted for 80% of the isolates when present with *E. faecalis* (Table 2). At 72h both *E. faecalis* and *L. bulgaricus* were recovered from the test tubes with *L. bulgaricus* accounting for 90% of the isolates (Table 2). At 7 days, *L. bulgaricus*

was the only isolate that survived when it was included in the mixture. In mixtures containing *E. faecalis* and no *L. bulgaricus*, *E. faecalis* was the only species that remained viable. Finally, in mixtures where neither *E. faecalis* nor *L. bulgaricus* were part of the mixture, *F. nucleatum* was recovered at 24 hours after mixture with the other Gram negative anaerobic bacteria (Tube 1); *E. alactolyticum* was recovered at both 24 and 72 hours after mixture with the remaining anaerobic bacteria (Tube 2). In tubes where *E. faecalis,* or *L. bulgaricus* were not part of the initial mixture, no bacterial species was recovered after 7 days (Table 2).

Discusssion

Mixtures of bacteria have been used in a few *in vitro* investigations.[9,10] Stuart *et al* tested the effect of Pulpdent®, a slurry of sterile water and $Ca(OH)_2$, formocreosol, and CMCP on a mixture of *S. mutans, A. viscosus,* and *B. gingivalis,* or *B. fragilis*.[9] They reported a significant reduction of the bacterial count by the test medications compared to the untreated controls. Only *S. mutans,* and *A. viscosus* were completely eliminated after treatment with Pulpdent®.

Estrela and associates used *M. luteus, S. aureus, F. neculeatum, P. aeruginosa, E. coli,* and *Streptococcus* spp. to prepare four different mixtures.[10] Because they did not have a negative control and did not determine the outcome when the same mixtures were used without treatment with $Ca(OH)_2$, the elimination of the bacteria cannot be attributed solely to the effect of this medication.

The purpose of the current study was to determine why two out of nine bacterial species survived in root canals of teeth after 1-week of inoculation.[11] This follow-up investigation involved using mixtures of seven of the nine bacterial species used in our

previous investigation in a PRAS broth (Anaerobic Systems) containing vitamin K, and hemin for one week. This time period was used to duplicate the time of inoculation of the root canals in our previous investigation.

To confirm bacterial interactions, seven bacterial species were mixed in test tubes in 16 different combinations (Table 1). Mixture I contained the three anaerobic gram-negative bacilli. In this mixture, we tested for gram-positive contaminants and the anaerobic system. Mixture II contained the three anaerobic gram-negative bacilli as well as the anaerobic gram-positive cocci (*E. alactolyticum)*. The purpose of this mixture was also to test procedures for growing the anaerobic bacteria. Mixture III had the anaerobic bacteria as well as *L. bulgaricus*. This mixture was incorporated to determine the effect of *L. bulgaricus* on the anaerobic bacteria. Mixture IV contained the anaerobic bacteria as well as the facultative anaerobe *E. faecalis.* This mixture examined the effect of *E. faecalis* on the anaerobic organisms. Mixture V contained all seven bacteria. Mixture VI contained the two gram-positive facultative organisms *E. faecalis,* and *L. bulgaricus.* This mixture was incorporated to demonstrate which facultative organism would overpower the other. Mixtures VII through XVI contained each bacterial species with either *E. faecalis,* or *L. bulgaricus.* Bacterial recovery and identification procedures showed that after a 7-day period, all bacterial species were killed, except for *L. bulgaricus*. These results were duplicated twice. In the mixtures where *E. faecalis* was mixed with other bacterial organisms and *L. bulgaricus* was not included in the mixture (mixtures 7, 9, 11,13, and 15), *E. faecalis* was the only organism that survived by the end of the 7-day mixture period.

Considering the results from our previous investigation, a slight modification to the composition of the bacterial mixture was made. *Prevotella denticola,* (ATCC 35308),

and *Campylobacter rectus* (ATCC 33238) were eliminated since they were not recovered beyond the first day after mixture. Furthermore, we had other representative bacteria from the same group—i.e. *P. denticola* and *P. buccae* which exhibited the same Gram staining, morphology, and oxygen requirements. In addition, because of the strict oxygen intolerance of these bacteria, they were more difficult to culture and would have complicated the microbiological procedures.

The results of our current experiments appear to be in conflict with those of our previous study.[11] In our previous experiment, *E. faecalis* was the only surviving bacteria isolated from dentinal shavings. In the current experiment, *L. bulgaricus* was the only surviving bacteria recovered from the test tubes. Recovering bacteria from the mixture at shorter time periods from test tubes revealed that *E. faecalis* survives in combination with *L. bulgaricus* for a period of three days. After 72 hours, *L. bulgaricus* was the only bacterial species that was able to survive in the mixture.

The difference between the results from the current study and those of our previous investigation might be due to the models used in these studies. *E. faecalis* may be able to penetrate the dentinal tubules more readily possibly due to its smaller size (0.5-0.8μm vs. 2μm x 8μm).[12] Because of their larger size, *L. bulgaricus* may not be able to penetrate the dentinal tubules where they can be shielded from medications.

Because in our previous experiment we recreated the smear layer, it is reasonable to assume that we entombed *E. faecalis* in the dentinal tubules. Since $Ca(OH)_2$ has been shown to have the ability to eliminate *Lactobacillus* spp.,[10] it seems plausible that *E. faecalis* would be the only bacterial species recovered from the root canal system. Combined with the fact that *E. faecalis* has been shown to survive up to 10 days without nutrition,[13] the survival ability of *E. faecalis* may have been due to the inaccessibility of

the $Ca(OH)_2$ into the dentinal tubules where the *E. faecalis* were residing, or alternatively, the $Ca(OH)_2$ was ineffective against *E. faecalis*.[10,13]

The acid that is produced by the *Lactobaccilus* spp. may have played a significant role in its survival ability in mixture with the other bacterial species, as well as its ability to outgrow other species.[14] Interestingly, several different *Lactobacillus* species are used in medicine as probiotics to rid certain patients of infections or conditions brought upon by an imbalance of the local environment.[15,16] In an *in vivo* randomized clinical trial, Cunningham-Rundles and associates administered *Lactobacillus plantarum* to children with human immunodeficiency virus (HIV) infections experiencing diarrhea and malabsorption associated with bacterial overgrowth. They reported that *L. plantarum* has the ability to colonize children with HIV and to elicit a specific systemic immune response after oral supplementation. [15] Gionchetti and colleagues, in a double-blind clinical trial, used oral bacteriotherapy as a maintenance treatment in patients with chronic pouchitis, an inflammation of the mucosa or inner lining of the ileoanal pouch. [16] Their results suggest that oral administration of viable lyophilized *Lactobacilli, bifidobacteria,* and *Streptococcus salivarius* is an effective treatment in preventing flare-ups of chronic pouchitis.

Our findings support the results of the studies that show that *Lactobacillus* spp. can outgrow other bacteria and can be used as a probiotic treatment. In our investigation, *L. bulgaricus* was able to overgrow and remain as the sole recoverable (surviving) bacterial species in the mixture.

There is yet another question that needs to be answered. *In vivo,* different anaerobic species are isolated when in mixture. In our *in vitro* experiment, why were *L. bulgaricus* the only species that survived in the test tube? Root canal infections are

typically polymicrobial in nature exhibiting 5-9 anaerobic bacterial species.[1-3] *Lactobacillus* spp. is a facultative species that is not generally found in such infections. In teeth that exhibit endodontic failture, *E. faecalis* is generally present as a mono infection. This may be explained by the results of our investigation, where in the absence of *L. bulgaricus*, *E. faecalis* was the only species that survived when in mixture with anaerobic bacteria.

Based on the results of this experiment, it appears that *E. faecalis* is able to survive in a test tube for three days when mixed with *L. bulgaricus*. *L. bulgaricus* was the only bacterial species capable of surviving more than three days in a test tube when mixed with other bacterial species. Bacterial interaction must be considered when using a mixture of bacteria in any *in vitro* experiment. The feasibility of using bacteria such as *Lactobacillus* spp. to compete with persistent bacteria such as *E. faecalis* should be investigated.

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Figure Legends

Figure 1. Identification of bacteria according to the procedural chart

Abbreviations

GPC—Gram-positive cocci

GPB—Gram-positive bacilli

GNB—Gram-negative bacilli

CHOC—Chocolate agar

PYR-pos—Pyruvate positive

IDS—Identification system

POS—Positive

NEG—Negative

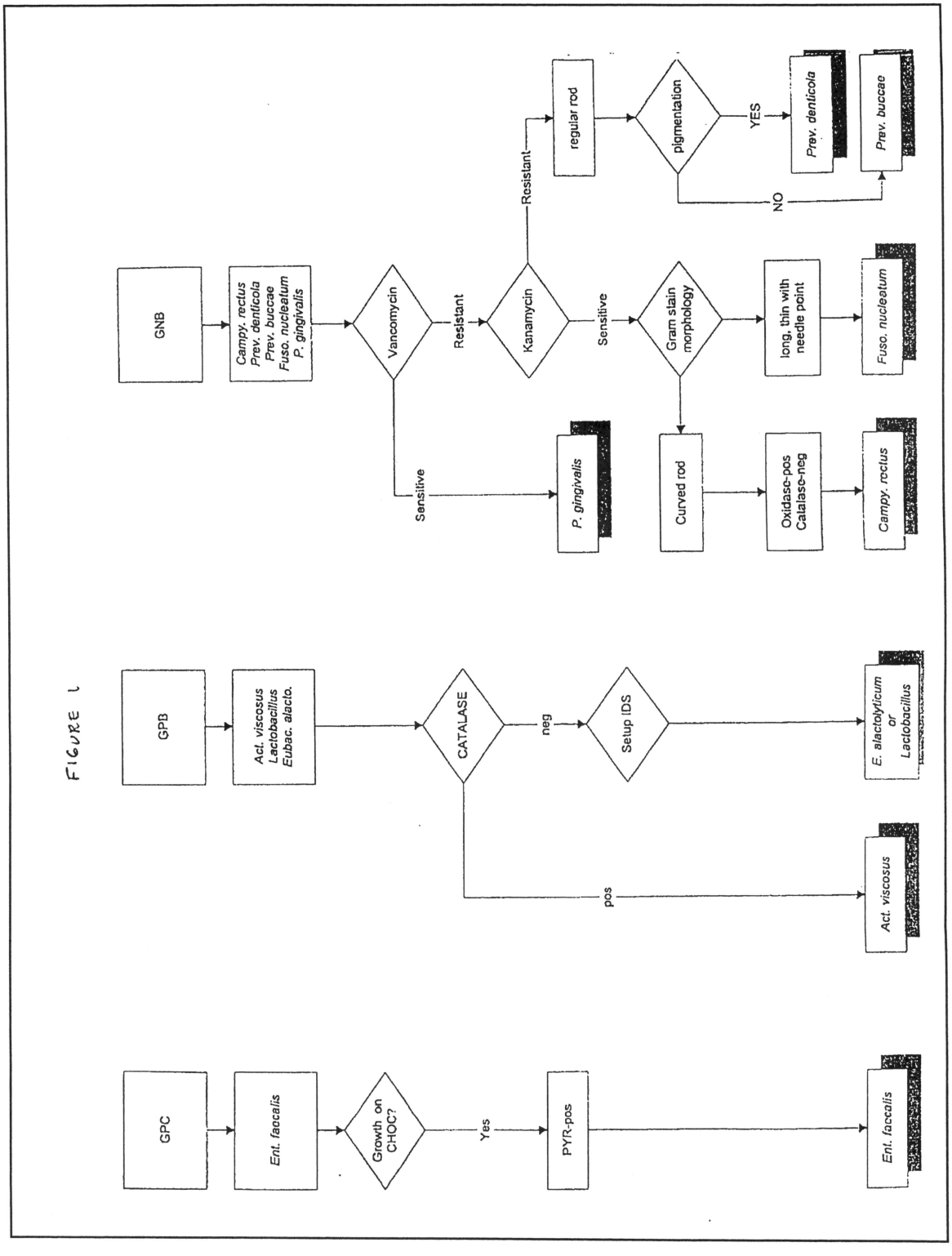

Figure 3-1—Identification of bacteria according to the procedural chart.

Table 3-1—Sixteen tubes containing different bacterial combinations

Bacterial Mixtures:
Tubes: 1. Three Gm- anaerobic bacilli--*P. buccae, P. gingivalis, F. nucleatum* 2. Four anaerobic bacilli—(Gm-)*P. buccae, P. gingivalis, F. nucleatum,* **AND** (Gm+) *E. alactyliticum* 3. Four anaerobic bacilli--*P. buccae, P. gingivalis, F. nucleatum, E. alactyliticum* **AND** *Lactobacillus sp.* 4. Four anaerobic bacilli--*P. buccae, P. gingivalis, F. nucleatum, E. Alactyliticum* **AND** *E. faecalis* 5. All seven bacteria 6. *Lactobacillus sp.* **AND** *E. faecalis* 7. *Eubacterium alactyliticum* **AND** *E. faecalis* 8. *Eubacterium alactyliticum* **AND** *Lactobacillus sp.* 9. *Prevotella buccae* **AND** *E. faecalis* 10. *Prevotella buccae* **AND** *Lactobacillus sp.* 11. *Fusobacterium nucleatum* **AND** *E. faecalis* 12. *Fusobacterium nucleatum* **AND** *Lactobacillus sp.* 13. *Porphyramonas gingivalis* **AND** *E. faecalis* 14. *Porphyramonas gingivalis* **AND** *Lactobacillus sp.* 15. *Actinomycosis viscosus* **AND** *E. faecalis* 16. *Actinomycosis viscosus* **AND** *Lactobacillus sp.*

Table 3-2—Bacterial species recovered from the original mixtures and their relative percentages

	T--zero	T—immediate	T--24 hours	T--72 hours	T--7 days
Tube 1	33.33% *P.b., P.g.,F.n.*	50%*F.n.,35%P.b.,15% P.g.*	30% P.b., 70% *F. n.*	No Growth	No Growth
Tube 2	25% *P.b., P.g., F.n., E.a.*	10% *F.n.*, 90% *E.a.*	100% *E. a*	100% *E. a*	100% *E.a*
Tube 3	20% *P.b., P.g., F.n., E.a., Lacto*	40% *F.n.*, 60% *Lacto*	100% *Lacto*	100% *Lacto.*	100% *Lacto*
Tube 4	20% *P.b., P.g., F.n., E.a., E.faecalis*	35% *P.b*, 65% *E.f.*	100% *E. f*	100% *E. faecalis*	100% *E. f*
Tube 5	14.3% *P.b.,P.g.,F.n.,E.a.,A.v.,E.f.,Lacto*	20%*Pb,15%Pg,*20%*E.f.,50%Lac*	25% *E.f., 75% Lacto.*	10% *E.f.,*90% *Lacto.*	100% Lacto
Tube 6	50% *E.f.,Lacto*	80% *Lacto.*, 20% *E.f.*	20% E.f., 80% *Lacto*	10% E.f., 90% *Lacto*	100% Lacto
Tube 7	50% *E.f. E.a.*	*25% E.a., 75% E.f.*	100% *E.f.*	100% *E.f.*	100 *E. f*
Tube 8	50% *Lacto.,E.a.*	*35% E.a., 65% Lacto.*	100% *Lacto.*	100% *Lacto.*	100 % *Lacto*
Tube 9	50% *P.b., E.f.*	10% P.b.,90% *E.f.*	100% E.f.	100% E.f.	100% *E. f*
Tube 10	50% *P.b., Lacto.*	50% P.b., 50% Lacto	100% *Lacto.*	100% *Lacto.*	100% *Lacto*
Tube 11	50% *F.n., E.f.*	50% F.n., 50% *E.f.*	100% *E.f.*	100% *E.f.*	100% E. f
Tube 12	50% *F.n., Lacto.*	50% *F.n., 50% Lacto*	100% *Lacto.*	100% *Lacto*	100% *Lacto*
Tube 13	50% *P.g., E.f.*	10% P.g.,90% *E.f.*	100% *E.f.*	100% *E.f.*	100% *E. f*
Tube 14	50% *P.g., Lacto.*	10% P.g., 90% *Lacto.*	100% *Lacto.*	100% *Lacto.*	100% *Lacto.*
Tube 15	50% *A.v., E.f.*	10% A.v.,90% *E.f.*	100% *E.f.*	100% *E.f.*	100% *E. f*
Tube 16	50% *A.v., Lacto.*	20% A.v., 80% *Lacto.*	100% *Lacto.*	100% *Lacto.*	100% *Lacto*

CHAPTER FOUR

DISCUSSION

The role of bacteria in endodontic infections is well established (Kakahashi *et al*, 1965; Moller *et al*, 1989). Mechanical instrumentation of the root canal greatly reduces the amount of bacteria; however, their eradication by mechanical cleaning and shaping is not possible (Bystrom and Sundqvist, 1981; Gomes *et al*, 1996; Dalton *et al*, 1998; Siqueira *et al*, 1999).

The need for a bactericidal irrigant used in conjunction of the chemomechanical cleaning and shaping is obvious. Sodium hypochlorite (NaOCl) has been the most widely used antibacterial irrigant in root canal therapy. Hand and colleagues (1978) demonstrated that a 5.25% solution was bactericidal, in addition, it exhibited properties of tissue necrosis. Furthermore, they showed that dilution of the recommended 5.25% solution significantly reduced its tissue necrosing ability. In 1981, Harrison and Hand demonstrated that dilution of 5.25% NaOCl also had a decrease in its bactericidal activity. This decrease was amplified in the presence of organic matter. Sodium hypochlorite has also been shown to inactivate endotoxin (Brady and del Rio, 1986).

In addition to bactericidal irrigants, intraappointment intracanal medicaments were needed for cases when root canal treatment could not be completed in one appointment (*e.g.* abscessed tooth with open apex). These intracanal medicaments ideally needed to be bactericidal, have inflammatory reduction properties, have neutralizing effects on tissue debris, have the property of inhibiting bacterial recontamination of the prepared root canal intraappointment, as well as having the ability to dry persistently wet canals (Grossman *et al*, 1986; Chong and Pitt Ford, 1992).

Morgan and coworkers (1991) tested the solvent effects of calcium hydroxide alone and with sodium hypochlorite on bovine pulp tissue. They reported that after 48 hours, there was no difference in using calcium hydroxide as a tissue solvent when compared to saline. Wadachi and co-investigators (1998) tested the effect of calcium hydroxide alone, and in combination with sodium hypochlorite for 1, 3, and 7 days. They reported that the tissue dissolving ability of sodium hypochlorite was superior even at only 30 seconds. However, they found that the best tissue dissolving ability to bovine root canal walls was when calcium hydroxide was used for 7 days, and sodium hypochlorite was used for at least one minute. These results were in agreement with Andersen *et al* (1991). These group of investigators also found that human pulp tissue solubility by calcium hydroxide required a time period of 7 days. Safavi and Nichols (1993) in an *in vitro* investigation showed that calcium hydroxide inactivates lipopolysaccharide, a component of gram-negative bacterial cell walls. Iodine Potassium Iodide (IKI) has also been recommended as an intracanal medicament due to the bactericidal activity of the halide Iodine (Safavi *et al*, 1985). They reported that IKI was better able to eliminate the bacteria in the dentinal tubules as compared to $Ca(OH)_2$. Iodine Potassium Iodide has not found its way to clinical practice, probably due to its potential for its allerginicity (Spangberg *et al,* 1973) and its potential to discolor teeth.

Ledermix®, a 50-50 mixture of triamcinolone (a corticosteroid) and demethylchlor-tetracycline (a tetracycline antibiotic), has also been suggested as an intracanal medicament (Schroeder, 1972; Heithersay, 1977). Although Ledermix® has found support for usage in Australia, it has not been commonly used in the united States.

Various time intervals have been investigated for the use of calcium hydroxide. In an *in vivo* investigation, Sjogren and colleagues (1991) used calcium hydroxide for 10

minutes and 7 days in teeth having necrotic pulps and exhibiting radiographic periapical lucency. They found that a 10-minute treatment was not sufficient to kill all bacteria, however; 7 days of calcium hydroxide treatment was able to render the canals bacteria free. Molander and coworkers (1999) used calcium hydroxide for two months in 50 teeth that showed persistent bacterial growth and reported that 20% of the teeth still exhibited bacterial growth. Orstavik and colleagues (1991) reported that after one week of calcium hydroxide dressing, 8 out of 23 root canals still exhibited viable bacteria. Siqueira and de Uzeda (1996) used calcium hydroxide mixed with saline or CMCP for 1hour, 1 day, and 1 week on bovine teeth infected with *Actonomyces israelii, Fusobacterium nucleatum,* and *Enterococcus faecalis*. They reported that calcium hydroxide, combined with CMCP effectively killed *A. israelii,* and *F. nucleatum* after 1 hour, and *E. faecalis* after one day. However; when mixed with saline, calcium hydroxide was ineffective against *F. nucleatum* and *E. faecalis* even after 1 week. Stevens and Grossman (1983) did an *in vivo* and *an in vitro* investigation of the bactericidal effect of calcium hydroxide on *streptococcus faecalis*. In the *in vivo* experiment, they infected the bacterial species into cat teeth, and used Pulpdent® (Pulpdent®) as a supernatant or in a slurry. They reported that when compared to CMCP, calcium hydroxide was not as effective against *S. faecalis.* The same results were obtained in the *in vitro* experiment where a small zone of inhibition was observed in petri dishes containing calcium hydroxide disks and infected with *S. faecalis.*

In the previous investigations, calcium hydroxide was either used *in vitro* against single bacterial species (Siqueira and de Uzeda, 1996; Orstavik and Hapaasalo, 1990; Berkiten, 2000; Perez *et al*, 1991; Abdulkadder, 1996) or two to three bacterial species in combination (Barrieshi *et al*, 1997; Stuart *et al,* 1991, Estrela *et al*, 1998). Few *in vivo*

investigations exist where the effect of calcium hydroxide has been tested against naturally occurring polymicrobial infections (Sjogren *et al*, 1991).

Although Siqueira and de Uzeda (1996) used *A. israelii, F. nucleatum* and *E. faecalis* to infect bovine teeth, they used the bacterial species separately. Orstavik and Hapaasalo (1990) used the single isolate, *E. faecalis*, to infect the bovine teeth in their investigation. Berkiten and coworkers (2000) infected 28 freshly extracted human teeth and infected two groups of teeth. They infected the first group with *S. sanguis*, and the other group with *P. intermedia*. They measured the distance of bacterial penetration into the dentinal tubules. Perez *et al*, 1996 infected freshly extracted bovine teeth with *S. sanguis*. They tested the effect of the smear layer on bacterial invasion. They concluded that the smear layer may act as a barrier for bacterial leakage. Abdulkader (1996) tested three different bacterial species as single isolates—*Capnocytophaga ochracea, Porphyromonas gingivalis,* and *Peptostreptococus micros.* He used four different calcium hydroxide containing root canal sealers and two calcium hydroxide root canal dressing materials on blood agar plates. He reported that Pulpdent® (Pulpdent®) showed the least effectiveness against the three organisms used by having the smallest zone of inhibition around *C. ochracea*, and no zone of inhibition around the two anaerobic bacteria used in the investigation.

Barrieshi and coworkers (1997) prepared a bacterial mixture of *F. nucleatum, P. micros* and *C. rectus*. They used the mixture to test coronal leakage after obturation and post preparation. They changed the bacterial suspension every 3 days to ensure bacterial viability. They noted bacterial leakage after 48 days and up to 84 days with *F. nucleatum and C. rectus* leaking to the apex. *Peptostreptococcus micros* was not detected at the apex of the teeth. Although this study was not testing the survival of the bacteria in mixture,

nor the effect of a medicament on the combination of bacteria, the authors noted that they changed the bacterial suspensions every 3 days. The reason for this was not given. Therefore, bacterial interaction was not addressed.

Stuart and colleagues (1991) mixed *S. mutans, A. viscosus,* and *B. gingivalis,* or *B. fragilis* and tested the effect of Pulpdent®, a slurry of sterile water and calcium hydroxide, formocreosol, and CMCP. After one hour of incubation, they sampled the teeth for bacteria. They report a significant difference in the reduction for all tested groups by all the test medications compared to the untreated controls. Although the investigators report such a significant reduction, they do not account for bacterial interaction. Only *S. mutans,* and *A. viscosus* were eliminated after treatment with Pulpdent®. The reported 99.9% reduction of *B. gingivalis* is practically meaningless since 0.01% remaining bacterial count translates to 1.61-100 x 10^3 bacteria remaining from the initial count of 1.61 – 100 x 10^6. Since the investigation was not carried out to any sufficient length of time, small number of bacteria would multiply to very large numbers in a short time. Finally, dentin shavings were not tested for bacteria to test presence of bacteria in dentinal tubules. Wang and Hume (1988) tested the effect of different acidic and basic materials on the pH of dentin hydroxyapetite. They showed that dentin chips and hydroxyapetite were extremely effective buffers for acids, but had a less buffering effect on NaOH. They concluded that because of the buffering of H^+ ions by hydroxyapetite, hydroxyl ions diffused less readily into the dentinal tubules. Therefore, bacteria may have infected the tubules, and not been eliminated by the effect of calcium hydroxide, especially after only one hour.

Estrela and coinvestigators (1998) used *Micrococcus luteus, Staphylococcus aureus, F. neculeatum, Pseudomonas aeruginosa, Escherichia coli,* and *Streptococcus sp.*

to make four mixtures. The investigators tested the effect of calcium hydroxide for periods of 0, 1, 2, 6, 12, 24, 48, 72 hours and 7 days. The bacterial species they used in their investigation were aerobic gram-positive cocci (*M. luteus, S. aureus,* and *Streptococcus sp.*), aerobic gram-negative coccus (*E. coli,* and *P. aeruginosa*), and anaerobic gram-negative rod (*F. nucleatum*). Their results showed that calcium hydroxide was effective in eliminating *M. luteus,* and *F. nucleatum* after 12 hours, *Streptococcus sp.* after 24 hours, *E. coli* after 48 hours, and *S. aureus* and *P. aeruginosa* after 72 hours. The investigators did not use controls in this experiment. They did not check to see what results would have been obtained with the same mixtures *without* treatment with calcium hydroxide. Therefore the elimination of the bacteria cannot be attributed to calcium hydroxide until such data are available.

Sjogren and colleagues (1991) tested the effect of short-term effect of calcium hydroxide for 10 minutes and 7 days. They used 30 single-rooted teeth having necrotic pulps and exhibiting radiographic periapical lucencies. After cleaning and shaping, they used calcium hydroxide for 10 minutes in 12 teeth, and for 7 days in 18 teeth. The root canals were initially sampled pre- and post-instrumentation under anaerobic conditions. The 10-minute samples were sealed with sterile foam for 1 week, and the 7-day samples were sealed with sterile foam for periods up to 5 weeks. At the initial sampling, the investigators noted the following strains: *F. nucleatum, B. denticola, E. faecalis, A. viscosus, B. buccae, P. micros, W. recta, E. alactolyticum, Lactobacillus sp.* in the root canals. Their results showed that 12 of thirty teeth exhibited no bacterial growth after mechanical cleaning and shaping that included the use of 0.5% sodium hypochlorite and endosonic files. Of the remaining nine teeth in the 10-minute group, six showed a persistence of the strains sampled initially. In the 7-day group, no bacterial growth was

found after seven days of calcium hydroxide treatment for up to five weeks. The investigators concluded that a 7-day treatment with calcium hydroxide renders the infected root canals bacteria free.

These investigators sampled only the root canal space for bacteria. However, removal of the smear layer was not mentioned. Neither was the dentin shavings sampled to see if bacteria were present in the dentinal tubules. It is plausible that bacteria may have been entombed in the tubules and blocked by the smear layer where the calcium hydroxide is less able to penetrate (Wang and Hume, 1988).

The above studies give conflicting reports on the efficacy of calcium hydroxide in eliminating bacteria from the root canal system (Molander *et al*, 1999; Haapasalo and Orstavik, 1987, 1990; Siqueira and de Uzeda, 1996, Barbosa *et al*, 1997; Stevens and Grossman, 1983; Estrela *et al*, 1998, Stuart *et al*, 1991, Abdulkader, 1996; Sjogren, 1991). The current investigation has ample support in the literature with its results that calcium hydroxide is ineffective against *E. faecalis.*

Only two *in vitro* investigations have tested the effect of calcium hydroxide on a mixture of a combination of bacteria (Estrala *et al*, 1998, Stuart *et al* 1991). Estrela and coinvestigators (1998) used *M. luteus, S. aureus, F. neculeatum, P. aeruginosa, E. coli,* and *Streptococcus sp.* to make four mixtures. The effect of calcium hydroxide was tested for periods of 0, 1, 2, 6, 12, 24, 48, 72 hours and 7 days. Their results showed that calcium hydroxide was effective in eliminating *M. luteus,* and *F. nucleatum* after 12 hours, *Streptococcus sp.* after 24 hours, *E. coli* after 48 hours, and *S. aureus* and *P. aeruginosa* after 72 hours. The investigators did not use a negative control group in this experiment. Therefore, the effect of bacterial interaction was not considered in the elimination of the bacteria. Although there was only one bacterial species common to this

and our investigation (*F. nucleatum)*, the results are in agreement if bacterial interaction is played a role in bacterial elimination. Stuart and colleagues (1991) mixed *S. mutans, A. viscosus,* and *B. gingivalis,* or *B. fragilis* and tested the effect of Pulpdent®, a slurry of sterile water and calcium hydroxide, formocreosol, and CMCP. They reported a significant difference in the reduction for all tested groups by all the test medications compared to the untreated controls. Only *S. mutans,* and *A. viscosus* were eliminated after treatment with Pulpdent®. They reported a 99.9% reduction of *B. gingivalis.* This reduction is effectively insignificant given the number of the initial bacterial count. In addition, dentin shavings were not tested for bacteria in dentinal tubules. This experiment was run for one hour, our own results show that there would have been sufficient bacterial interaction taking place. Given these results, data from this experiment would be in agreement with our own if bacterial interaction was considered to playing a role in the elimination of bacteria.

Furthermore, only two studies tested the effect of calcium hydroxide treatment at times less than 7 days (Estrela *et al*, 1998, and Sjogren *et al*, 1991). Estrela and colleagues (1998) tested the effect of calcium hydroxide on six bacterial strains *in vitro* for 0, 1, 2, 6, 12, 24, 48, 72, and hours and 7 days. Sjogren and coworkers tested the effect of calcium hydroxide *in vivo* for 10 minutes and 7 days. Estrela and colleagues showed that calcium hydroxide was effective in eliminating eliminating *M. luteus,* and *F. nucleatum* after 12 hours, *Streptococcus sp.* after 24 hours, *E. coli* after 48 hours, and *S. aureus* and *P. aeruginosa* after 72 hours. Again, these results would be in agreement with our own if bacterial interaction is considered as playing a role in the bacterial elimination. Sjogren and colleagues (1991) showed that calcium hydroxide was not effective in eliminating the bacteria from the canals in a 10-minute period, but a 7-day treatment was

effective. Our results do agree with the results of Sjogren and his coworkers. However; since the investigators did not consider to test any presence of bacteria in the tubules, we cannot accept the statement that calcium hydroxide was able to eliminate all bacteria from the root canal.

Considering the available research on the effect of calcium hydroxide on a combination of bacteria, and different time periods, we decided to mix a combination of nine representative bacteria. The bacterial species that were investigated in this study have previously been sampled from root canals with endodontic infections (Sjogren *et al*, 1991; Sundqvist, 1976).

The bacterial strains that were chosen were representatives of the following categories: facultative gram-positive coccus (*E. faecalis)*, facultative gram-positive bacilli (A. viscosus, *Lactobacillus sp.)*, anaerobic gram-positive bacillus (*E. alactyliticum*), anaerobic gram-negative rods (*P. gingivalis, C. rectus, P. denticola,* and *P. buccae*), and anaerobic gram-negative fusiform bacteria (*F. nucleatum*). The test periods for the calcium hydroxide treatment was chosen for 20 minutes, 1, 2, 3, 4, 5, 6, 7 days. In their *in vivo* investigation, Sjogren and colleagues (1991) reported that a 7-day treatment of calcium hydroxide was able to eliminate all bacteria, yet the 10-minute treatment was not. Our aim was to test the effect of calcium hydroxide at time periods that fell between the 10 minutes and 7 days.

The results of part I of our experiment is in agreement with the results of Molander and colleagues (1999), Orstavik and Hapaasalo (1990), Siqueira and de Uzeda (1996), Barbosa and coworkers (1997); Stevens and Grossman (1983), Estrela and coworkers (1998), and Abdulkader (1996). All of these investigators have shown that calcium hydroxide to be ineffective against *E. faecalis*. Furthermore, our results also

compares well with the results of Barbosa and coworkers (1994) who demonstrated that *Lactobacillus sp.* was killed in approximately three minutes when in direct contact with calcium hydroxide. Our results showed that although *Lactobacillus sp.* was recovered in nine out of 10 samples at the end of 20-minute treatment, there were no remaining *Lactobacilli* by the 24-hour treatment time of calcium hydroxide.

Although it may seem that our results do not agree with results reported by Sjogren and colleagues (1991), they appear to actually do. They reported that there were no viable bacteria found in the root canals after a 7-day treatment with calcium hydroxide. Our results are in agreement with this. However; Sjogren and coworkers did not test the dentin shavings from those canals to see if any viable bacteria exist in the dentinal tubules. Because they had the teeth sealed for a period of up to five weeks, they apparently assumed that if there were bacteria in the tubules, a positive culture would have been obtained. However, since the smear layer was not removed, bacteria may have been entombed in the tubules where calcium hydroxide has a lesser chance of reaching the bacteria.

This investigation appears to be one of the first that considers bacterial interaction as a major mechanism in the elimination of microbes in mixtures *in vitro*. The two investigations that have used a combination of three bacterial species in mixture to test the effects of calcium hydroxide (Estrela *et al*, 1998; Stuart *et al*, 1991) did not consider bacterial interaction as a possible explanation for their results. Estrela and coworkers (1998) did not have a negative control group. Therefore, they could not have known whether their results were due to solely the effect of calcium hydroxide or a combination of bacterial interactive effects as well as the effect of the treatment. Although Stuart and colleagues (1991) incorporated control teeth that received no calcium hydroxide

treatment, they did not measure the effect of bacterial interaction on their results. Although there was a significant reduction in the amount of bacteria in their samples, they noted a total elimination in only two of four bacterial species from the root canal. Furthermore, they did not sample dentin shavings to determine whether the dentinal tubules were void of viable bacteria.

Our investigation used several levels of controls to ensure that any bacterial reduction/elimination was due to the treatment of the calcium hydroxide. We were able to determine this by mixing the bacteria and re-isolating them in the test tube after different time periods.

The results of the calcium hydroxide treatment during the different time periods seemed as though all bacterial species in the experiment were eliminated except for *E. faecalis*. These results are in accordance with other investigations (Barbosa *et al*, 1994, Bystrom *et al*, 1985, Molander *et al*, 1999). Barbosa and coworkers (1994) showed that calcium hydroxide killed many of the strains used in their investigations within hours. However, they showed that *E. faecalis* was not eliminated by calcium hydroxide treatment.

Part II involved mixing the same bacterial species in a reduced anaerobic broth containing vitamin K, and heme for two weeks and recovery of the original bacterial species. One week's mixture time was used to duplicate the time of inoculation of the root canals. If the nine bacteria were recovered, then the bacteria killed by the treatment could be attributed to effect of the $Ca(OH)_2$. If on the other hand, results showed that some bacteria are not recovered, then further investigation would need to be performed to explain the results.

In order to confirm bacterial interactions, the seven bacterial species were mixed in a test tube and bacterial recovery and identification procedures showed that after a 7-day period, all bacterial species were killed, except for *Lactobacillus bulgaricus*. These results were repeated and verified twice. The results of the two experiments were in conflict. In the initial experiment, *E. faecalis* was the remaining bacterial isolate recovered from dentinal shavings. In the follow-up experiment, *Lactobacillus sp.* was the remaining bacterial isolate recovered from the test tubes. These conflicting results required an explanation.

Further testing of the bacterial mixture in the test tube revealed that *E. faecalis* survives in combination with the *L. bulgaricus* for a period of three days. By the fourth day, *L. bulgaricus* is the only bacterial species that survives.

One feasible explanation was that *E. faecalis*, during the first 3 days in mixture was penetrating the dentinal tubules much more readily than the other bacteria. *L. bulgaricus* was the main competitor of *E. faecalis* vying for dentinal tubule penetration. *E. faecalis* has a size of 0.5-0.8μm (Bergey's Manual). Furthermore, *E. faecalis* is a coccus that would have an easier time penetrating the tubules which range from 2-3.2μm in diameter at the pulpal wall (Garberoglio and Brannstrom, 1976). *Lactobacillus bulgaricus*, on the other hand is a bacillus that has the size of 2μm x 8μm (Bergy's Manual). As well, the *Lactobacillus* spp. are bacteria that are found in chains, making it more difficult for them to penetrate the tubules. Therefore, it would make sense that the smaller *E. faecalis* cocci would have an easier time penetrating the dentinal tubules than the *Lactobacillus sp.* which are larger bacilli in chains.

Since in part I of the experiment the smear layer was recreated to entomb the bacteria in the dentinal tubules, it follows that *E. faecalis,* which had penetrated the

dentinal tubules would be entombed there. Also, since calcium hydroxide has been shown to kill all other bacteria, it seems plausible that calcium hydroxide would be able to kill the bacteria—*Lactobacillus bulgaricus*—in the root canal. However; since the *E. faecalis* was inaccessible to the calcium hydroxide, *or* the calcium hydroxide was ineffective against *E. faecalis*, it was recovered, isolated and identified even after 7 days of calcium hydroxide treatment.

In the follow-up experiment—carried out in test tubes—since there were "no tubules" for the *E. faecalis* to penetrate. *E. faecalis* was unable to avoid interaction with the *Lactobacillus sp. E. faecalis* was overpowered by the *Lactobacillus* spp. which was the only isolate that survived after 7 days of all the bacteria in mixture.

It is worthy to note that *in vivo, Lactobacillus* spp. is not a predominant isolate in endodontic infections. There are several possible explanations for this. The root canal system has an intricate canal morphology rather than being perfect cylindrical tubes as in this experiment. Therefore, some bacteria may avoid interaction with other bacteria. Furthermore, the microenvironment plays a major role in determining the bacterial make-up of an endodontic infection. In addition, the immune system also plays a dominant role in contributing the make up of infections.

Interestingly, several different *Lactobacillus* species are used in medicine as probiotics to rid certain patients of infections or conditions brought upon by an imbalance of the local environment (Cunningham-Rundles *et al*, 2000; Gionchetti *et al*, 2000). In an *in vivo* randomized clinical trial, Cunningham-Rundles and associates administered *Lactobacillus plantarum* to children with human immunodeficiency virus (HIV) infections experiencing diarrhea and malabsorption associated with bacterial overgrowth. They reported that *Lactobacillus plantarum* had the ability to colonize children with HIV

and to elicit specific systemic immune response after oral supplementation. Gionchetti and colleagues (2000) reported in a double-blind clinical trial where oral bacteriotherapy was used as a maintenance treatment in patients with chronic puchitis, an inflammation of the mucosa or inner lining of the ileoanal pouch. Their results suggest that oral administration of viable lyophilized bacteria of *Lactobacilli, bifidobacteria,* and *Streptococcus salivarius* was an effective treatment in preventing flare-ups of chronic pouchitis.

The ability of *Lactobacillus sp.* to overgrow other bacteria as evidenced by its usage in medicine as a probiotic supports our own results. In our investigation, *Lactobacillus sp.* was able to overgrow and remain as the only recoverable (surviving) bacterial species in our mixture.

Conclusion

Based on our results, it appears that the use of calcium hydroxide in canals infected with *E. faecalis* is ineffective. A new method or material is needed to eradicate bacteria such as *E. faecalis* in a single appointment for the benefit of both patient and practitioner. Furthermore, our results from the follow-up experiment showed that *E. faecalis* survived up to three days when in mixture with *Lactobacillus* spp. and other bacterial spp. *Lactobacillus* spp. was the only bacterial species able to survive in a test tube when in mixture with other bacterial species for a period of over three days. Further studies are needed to explore the feasibility of using bacteria such as *Lactobacillus* spp. to compete with persistent bacteria such as *E. faecalis* to the advantage of the patient.

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