Contact guidance of endodontic pathogens on micro-grooved polymethylmethacrylate discs

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Dedication

I dedicate this thesis to my wife Ilisapeci Viwa Lailai, for her unconditional love, support and patience.
Abstract

Bacteria are the primary cause of pulp and periapical pathology. They gain entry into the tooth through exposed dentinal tubules. Although bacteria have been shown to invade these dentinal tubules, the cell interactions or processes involved in invasion are still not clear.

This study will look at one possible process, contact guidance/thigmotropism, that has been shown in plants, some animal cells and with a known oral microbe *Candida albicans.*

**Aim:** To determine contact guidance characteristics by endodontic pathogens *Streptococcus mutans* NG8, *Streptococcus gordonii* DL-1, *Enterococcus faecalis* JH2-2 and *C. albicans* ATCC 10261 on micro-grooved polymethylmethacrylate (PMMA) discs. In addition, to determine the effect an amino acid sequence in collagen type I (Glycine-Proline-Alanine) has on growth characteristics. **Methods:** Micro-grooves of various dimensions (2 µm x 2 µm x 2 µm (Width Depth Spacing), 2 µm x 2 µm x 4 µm (WDS), 4 µm x 2 µm x 2 µm (WDS) for bacteria and 4 µm x 4 µm x 2 µm (WDS), 4 µm x 4 µm x 4 µm (WDS) for *Candida* were produced on PMMA to simulate some characteristics of dentinal tubules. Blank discs were used as a control. Discs were inoculated with collagen type I overnight then incubated in mono-solutions of each test organism; 2 days for bacteria and 3 hours for *Candida* followed by processing discs for scanning electron microscopy (SEM). Images were captured using SEM micrographs. The methods were repeated with Glycine-Proline-Alanine inoculated into the mono-solutions. **Results:** Qualitative analysis demonstrated that *C. albicans* ATCC 10261 hyphae when incubated on blank PMMA discs did not exhibit contact guidance/thigmotropism. In contrast *C. albicans* ATCC 10261 hyphae exhibited contact guidance along grooves and edges on grooved substrate. In comparison, the bacteria *S. gordonii* DL-1 and *S. mutans* NG8 showed some evidence of contact guidance by chain growth along grooves while *E. faecalis* JH2-2 gave no indications of exhibiting contact guidance. Glycine-Proline-Alanine affected cell adhesion and length of chaining growth in *S. gordonii* DL-1 and *S. mutans* NG8, and hyphae formation in
C. albicans ATCC 10261. The Glycine-Proline-Alanine had no effect on E. faecalis JH2-2. **Conclusion:** The results suggest that contact guidance could be involved in microbial invasion of dentine.
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Philip Alick Ilai-Alaia Lailai-Tasmania my pride and joy in my life.

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<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>Als</td>
<td>Agglutinin-like sequence</td>
</tr>
<tr>
<td>AS</td>
<td>Aggregation substance</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
</tr>
<tr>
<td>BHY</td>
<td>Brain Heart Yeast extract</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>Chit</td>
<td>Chitinase</td>
</tr>
<tr>
<td>CSP</td>
<td>Competence stimulating peptide</td>
</tr>
<tr>
<td>CWP</td>
<td>Cell wall protein</td>
</tr>
<tr>
<td>DEJ</td>
<td>Dentine enamel junction</td>
</tr>
<tr>
<td>DES</td>
<td>Discontinuous edged surfaces</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Esp</td>
<td>Enterococcus surface protein</td>
</tr>
<tr>
<td>FAs</td>
<td>Focal adhesions</td>
</tr>
<tr>
<td>FESEM</td>
<td>Field-emission scanning electron microscope</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>Gly-Pro-Ala</td>
<td>Glycine-Proline-Alanine (tripeptide)</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidyl inositol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GSB</td>
<td>Glucose salts biotin</td>
</tr>
<tr>
<td>HGF</td>
<td>Human gingival fibroblasts</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour</td>
</tr>
<tr>
<td>Lip</td>
<td>Lipase</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>NaOCl</td>
<td>Sodium hypochlorite</td>
</tr>
<tr>
<td>NZDS</td>
<td>New Zealand Dermatological Society</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDL</td>
<td>Periodontal ligament</td>
</tr>
<tr>
<td>PIR</td>
<td>Protein with Internal Repeats</td>
</tr>
<tr>
<td>Pl</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethylmethacrylate</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SAG</td>
<td>Salivary agglutinin glycoprotein</td>
</tr>
<tr>
<td>Sap</td>
<td>Secreted aspartyl proteinase</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope / microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
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</table>

xiii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSBY</td>
<td>Tryptic soy broth with yeast extract</td>
</tr>
<tr>
<td>WDS</td>
<td>Width depth spacing</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>YEPD</td>
<td>Yeast extract peptone dextrose</td>
</tr>
<tr>
<td>μm</td>
<td>Micro-meter</td>
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1 INTRODUCTION

Bacteria are present in high numbers in the oral cavity (Aas et al. 2005; Figdor & Sundqvist 2007) and it is well established that bacteria are the major cause of pulp and periapical disease (Kakehashi et al. 1965; Sundqvist 1976; Möller et al. 1981; Fabricius et al. 1982). Pulp and periapical disease may result in a range of clinical symptoms ranging from a mild sensitivity to hot stimuli to pain when eating, sleepless nights and facial cellulitis - a sign of spreading infection (Sigurdsson 2003; Abbott 2007).

Bacteria can invade the tooth structure through the fluid filled dentinal tubules (Love 1996b, 2002). The density of dentinal tubules in dentine varies from ~15,000 to ~20,000 mm$^2$ at the dentinal-enamel junction to ~45,000 to 65,000 mm$^2$ at the pulp/dentine interface (Garberoglio & Brännström 1976; Pashley 1990). There are several pathways by which bacterial cells invade exposed dentine/dentinal tubules including carious lesions, compromised restorations, tooth wear, cracks and dental trauma (Tronstad & Langeland 1971; Pashley 1990; Peters et al. 1995; Love 1996a).

The exact mechanisms by which bacteria move or propagate through the dentinal tubules is still not clear, though up regulation of cell surface adhesins, bacteria-host interactions, and chain-growth have been identified as mechanisms (Love 2002).

It is well recognized that collagen is the major dentine structure to which bacteria adhere. Collagen binding and binding of proline rich-proteins is based on the dentine invasion/collagen interaction model by Love et al. (1997). They demonstrated that bacterial binding to immobilised collagen was central to tubule invasion and that free collagen competitively reduced dentinal tubule invasion by Streptococci in vitro.

Contact guidance or thigmotropism has been shown to be exhibited by a number of eukaryotic cells including the hyphae produced by the fungus Candida albicans in vitro by Sherwood et al. (1992) and Gow et al. (1994a) later
confirmed by Sen et al. (1997a). As a result of this contact guidance by C. albicans hyphae, the filamentous projections from yeast cells grow along firm surfaces, along scratches in a membrane or ridges on a polystyrene disc and penetrate through holes in membranes (Gow et al. 1994b).

If bacteria that invade dentine also show contact guidance then understanding the exact mechanisms of this property is essential to developing a novel approach that may prevent the initial adhesion to tooth substrate and propagation of bacteria into and within the dentinal tubules.

Research into whether the bacteria in this proposed study, Enterococcus faecalis JH2-2, Streptococcus mutans NG8 and Streptococcus gordonii DL-1 exhibit contact guidance has not been documented in the literature. A pilot study was undertaken by undergraduate student Nadell Ibriahim, at the School of Dentistry to investigate contact guidance by bacteria.

A major breakthrough since then has been the manufacturing of plastic discs with grooves 2 µm wide compared to the larger 10 µm grooves used by Ibriahim. Therefore it is now possible to test various combinations of grooves, spacings, and surface coatings e.g collagen, fibronectin that resemble more closely the dentinal tubules.

In addition, a tripeptide Glycine-Proline-Alanine will be examined to determine whether it affects bacterial growth or binding; particularly chaining growth. This tripeptide has been demonstrated to inhibit dentine invasion and chaining growth of bacteria in controlled concentrations by Love (unpublished results).
2 LITERATURE REVIEW

2.1 Thigmotropism

Thigmotropism also known as contact sensing or contour-guidance is the ability of an organism or a cell to sense and respond to topographical changes. This response may either be guided by grooves, ridges, scratches or pores in the topography. A number of organisms have been shown to exhibit thigmotropism in vitro that includes plant tendrils and roots, chondrocytes, endothelia, epithelia, fibroblasts, leucocytes, lymphocytes, macrophages, mesenchyme, neurons, osteocytes, oligodendrocytes, smooth muscle cells, some tumour cells and fungi (Sherwood et al. 1992; Gow et al. 1994a; Curtis & Wilkinson 1997).

2.2 Thigmotropism in plants

Plants’ thigmotropic characteristics permits them to survive in their respective environments, for example clinging to supporting structures avoiding barriers, and changing morphology (Jaffe et al. 2002). Thigmotropism can be seen in highly specialised structures known as tendrils. Many plants that do not have strong trunks use tendrils for support allowing continual growth towards a light source (Darwin 1865). Tendrils are long, slender organs often derived from either modified stems, leaves or flower peduncles. Tendrils are most sensitive to touch. The tendril rapidly coils around a structure as soon as direct contact occurs, while staying in close contact with the contacted object. The ventral side undergoes contraction while the dorsal side undergoes growth allowing coiling around the object (Jaffe & Galston 1966, 1968).

Thigmotropism is not only limited to tendrils but also exhibited in plant roots. Plant roots respond to several cues to maintain growth such as seeking out nutrients and water source, gravity and touch. Plant roots under normal conditions grow down vertically through soil by the process known as gravitropism (Monshausen et al. 2008). When a plant root encounters a barrier
or obstacle such as a rock, the root will circumnavigate the obstacle using touch guidance (Massa & Gilroy 2003). For example, an Arabidopsis thaliana plant root forms a step-like structure when the root tip encounters a barrier. Initial bending of the root occurs at the central elongation zone above the root tip when a barrier is encountered, followed by a second bend at the distal elongation zone just behind the root tip. As the root tip transverses the surface of a barrier the tip tracks along the surface of the obstacle. Gravitropism continues once the obstacle is cleared (Massa & Gilroy 2003).

The exact mechanism of thigmotropism in plants is not clear, however theories have been proposed: cellular signalling (calcium, jasmonates, ethylene, abscisic acid, auxin, brassinsteroids, nitric oxide and reactive oxygen species) and mechanosensing (rapid response and response over time) (Chehab et al. 2009; Monshausen & Gilroy 2009).

2.3 Thigmotropism by animal cells

Several selected cells have been used to study thigmotropic responses to topographical cues, with each responding to different dimensions. Cell types studied in an in vitro setting include chondrocytes, endothelia, epithelia, fibroblasts, leucocytes, lymphocytes, macrophages, mesenchyme, neurons, osteocytes, oligodendrocytes, smooth muscle cells, some tumour cells and fungi (Curtis & Wilkinson 1997). The cells used to conduct these experiments were from animals such as poultry, porcine and bovine cells. Few studies have used human oral cells to study responses to selected topographies (Oakley & Brunette 1995; Hamilton & Brunette 2005; Hamilton et al. 2009; Hamilton et al. 2010; Lavenus et al. 2011).

An earlier study, using human gingival fibroblasts (HGF) grown on smooth surfaces exhibited a spreading pattern with a protruding leading edge (Oakley & Brunette 1995). This protruding edge is described as a lamellopodia. The lamellopodia may also have protrusions known as filopodia that have been suggested to propel the cell forward in periodontal ligament fibroblasts ex vivo.
Cell movement occurs when the leading edge elongates followed by cell contraction, allowing the cell to propel forward (Hamilton et al. 2010).

Human periodontal ligament (PDL) fibroblasts grown on glass slides in vitro exhibit no preferred direction of growth on smooth surfaces. The cells were more rounded and demonstrate a spreading effect when viewed under scanning electron microscopy (SEM). In comparison, on grooved surfaces cells have an elongated morphology and migrate parallel to the groove. In addition grooves, discontinuous grooves and smooth topographies had no significant differences in PDL cell migration velocity (Hamilton et al. 2010).

In contrast, Hamilton and Brunette (2005) reported that HGF align along titanium-coated discontinuous edged surfaces (DES), which were comprised of repeated open square boxes with gaps at each corner. The HGF aligned diagonally in the DES boxes and passed through to the next DES box. This demonstrates that a continuous edge is not a prerequisite to guide cells in a specified direction.

However, the substrates’ surface properties may have influenced the characteristics of the cells, such as wettability, chemical composition and surface roughness. HGF exhibit a significantly higher number of focal points on titanium when compared to tissue culture polystyrene and glass substrates. This may have caused cells to be more spindle-like shaped and branched than round when grown on smooth surfaces. This suggests that the behaviour of HGF may be modulated by specific substrates (Lavenus et al. 2011).

The mechanism of contact guidance on grooved topographies remains controversial, but likely involves focal adhesions (FAs), cytoskeletal arrangements and microtubules (Hamilton et al. 2009).
2.4 Thigmotropism by *Candida*

A number of studies have shown that the oral fungus *C. albicans* exhibits thigmotrophic cues to several substrates including grooves, ridges, scratches and pores in the substrate (Sherwood *et al.* 1992; Gow *et al.* 1994a).

Gow and associates (1994b) demonstrated *in vitro* the touch-sensitive responses of *C. albicans* hyphae when grown on selected membrane surfaces. *C. albicans* hyphae were shown to grow along cellophane membrane surfaces in a straight line. When hyphae encountered a scratch or grooved surface, the tip aligned itself parallel to the change in the topography (scratch or groove) and continued to grow alongside the scratch or groove. The scratched surface was made using sandpaper. Similarly in deeper grooves several hyphae were shown to reorientate and lie parallel along the same groove. However, hyphae were also observed crossing over the grooves while not in direct contact with the surface of the cellophane membrane when viewed using stereo-imaging. In addition, hyphae growth was also shown to stay in close contact and be guided by polystyrene ridges. This behaviour exhibited by the hyphae suggests that there is an interaction between the hyphae and the change in the surface topography.

It has also been shown that *C. albicans* hyphae reoriented their direction of growth to invade nuclepore filters. Nuclepore filters have an irregular distribution or pattern of circular pores, and the edge of the pore forms a 90° angle with the internal structure of the pore that transverses the filter. *C. albicans* hyphae invaded a filter, via the pore, whenever the hyphae come into contact with an edge. The hyphae stayed in close contact with the surface when growing over the edges and grew both towards and away from the nutrient medium. As such, this behaviour exhibited by *C. albicans* may be a response to the change in the topography substrate and not through chemotropism; that is towards a nutrient medium. In addition hyphae that were noted extending over a groove were not in direct contact with the substrate (Sherwood *et al.* 1992; Gow *et al.* 1994a).
Recent work using *C. albicans* mutants lacking in certain proteins are less able to penetrate oral epithelial cells *in vitro* and mice kidney tissue during systemic infection *in vivo* (Brand *et al.* 2008). *C. albicans* mutants defective in the protein GTPase Rsr1p (gene *RSR1*) and GTPase-activating protein Bud2p (gene *BUD2*) have been identified to be associated with hyphal guidance (thigmotropism), maintaining cell polarity and directed cell growth *in vitro* (Hausauer *et al.* 2005). The deletion of the genes *RSR1* and *BUD2* reduces the number of hyphal filaments penetrating monolayers of oral epithelial cells *in vitro* by 44% and 68% respectively. Histological Periodic acid-Schiff stains of kidney tissue in mice demonstrated the parent strain hyphae had a more diffuse and extensive infiltration, compared to the *RSR1* deleted gene strain had more localized, focal accumulations containing abnormal *candida* forms. Tissue invasion *in vivo* by *Candida* may be augmented by *RSR1* during a systemic infection (Brand *et al.* 2008).

### 2.5 Oral yeast species

Yeast are part of the oral cavity’s normal commensal flora, with *C. albicans* being the most dominant oral yeast species (Egan *et al.* 2002; Waltimo *et al.* 2003). Egan and colleagues (2002) identified yeasts being the most recovered isolates from saliva (19/59 samples or 32.2%) and root canal samples, with the majority belonging to the genus *Candida* (29% of patients). Of these *C. albicans* was the most prevalent (17/23 patients). Other species have also been identified such as *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, *C. kefyr*, *C. parapsilopsis* and *C. dubliniensis* (Odds 1988; Nastri *et al.* 2011).

Although *Candida* is generally accepted as a normal oral commensal the prevalence in primary endodontic infections and persistent/secondary endodontic infections is very low. The various techniques used to assess the presence of yeast in root canals [SEM, polymerase chain reaction (PCR), light microscopy (LM) and transmission electron microscopy (TEM)] all show similar results in detecting the presence of yeasts *in vivo* (Siqueira & Sen 2004). Therefore the
role that *Candida* may have in the cause or progression of endodontic infections is still not known.

### 2.6 Candida albicans

#### 2.6.1 General characteristics

According to the New Zealand Dermatological Society (NZDS) 2011, *Candida* species are present in 50% of healthy mouths.

*Candida* is a eukaryotic organism belonging to the fungus kingdom. There are three main groups (phyla) of fungi, namely *Zygomycota, Basidiomycota* and *Ascomycota*. The majority of fungi associated with human diseases are *Ascomycetes* from the subphyla *Saccharomycotina*, which includes the genus *Candida* (Fitzpatrick et al. 2006).

The *Candida* genome is within a nucleus that is surrounded by a membrane. The membrane is continuous with the endoplasmic reticulum and cell contents are within a rigid cell wall composed mainly of glucan (β(1,3)- and β(1,6)- chains of glucose), chitin (linear polymer of β(1,4)-N-acetyl glucosamine) and mannan (Hoog & Guarro 1995; De Groot et al. 2007). The inner layer is covalently linked by chitin polysaccharides and glucan provides strength and rigidity. The outer cell wall is enriched with mannoproteins that provide the wall with its fibrillar structure when viewed under SEM (Munro 2010).

*C. albicans* grows primarily as ovoid yeast cells which have the potential to grow pseudohyphae and hyphae in response to environmental changes (Gow et al. 2002; Sudbery et al. 2004; Whiteway & Bachewich 2005). This morphological switch may be triggered by interactions with other *C. albicans* cells and bacterial cells (Shareck & Belhumeur 2011). The change from yeast to hyphal morphological state is believed to be involved in the pathogenesis of *C. albicans* (Sherwood et al. 1992; Gow et al. 1994b).
C. albicans is considered to be an opportunistic pathogen because of its nature of becoming pathogenic when the host-fungus interaction becomes unbalanced in the favor of the fungi. The fungus is then able to initiate infection and cause disease. For example, patients with acquired immune deficiency syndrome (AIDS) are immuno-compromised and prone to oral and oesophageal candidiasis (thrush) when compared to normal healthy individuals (MacCallum 2010).

2.6.2 Candida albicans pathogenesis

Pathogenesis is the micro-organism’s ability to infect the host and produce disease, resulting from host-pathogen interactions favouring the pathogens. The determinants of pathogenicity are called virulence factors.

Candida infection develops when the host has been compromised through certain factors. These factors may include host physiologic status (normal or pathologic changes), dietary (high carbohydrate diet), mechanical (denture wearing) and iatrogenic factors such as broad-spectrum antibiotics and corticosteroids (Odds 1988). This will provide the environmental conditions and nutrients which are essential for attachment and growth (Waltimo et al. 2003).

The pathogenesis of Candida species and in particular C. albicans, is dependent on several virulence factors which are (1) adaptability to a variety of environmental conditions, (2) adhesion to variety of surfaces through production of cell wall-associated surface adhesion/recognition molecules, (3) production of hydrolytic enzymes, (4) morphologic transition and invasion, (5) evading host defences and (6) dentine colonization (Slutsky et al. 1985; Sheperd 1992; Calderone & Fenzi 2001; Gow et al. 2002; Nather & Munro 2008; Naglik & Hube 2010). The virulence factors associated with thigmotropism will be discussed including (2), (4) and (6) respectively.

2.6.2.1 Adhesion (surface adhesion/recognition molecules)

Adhesion is an important stage that must occur before invasion of the host tissues. C. albicans cell wall proteins (CWP) enable adhesion to host cells. Three
different groups of proteins have been identified. The most abundant CWP are glycosylphosphatidyl inositol (GPI)-anchored proteins, which are covalently attached and either are permanently localized in the membrane or translocated out to the cell wall. The GPI-proteins are synthesized and processed in the endoplasmic reticulum (Mao et al. 2008). The second group of protein, termed Pir (proteins with internal repeats) has previously been identified (Kandasamy et al. 2000; Kapteyn et al. 2000) and the third type of CWP protein which lack the covalent attachment to the polysaccharide matrix, are either cell wall associated or secreted from the cell (secretory proteins) (Chaffin 2008).

The GPI-CWP have been extensively researched in the 1990s and this identified several _C. albicans_ adhesins (e.g. agglutinin-like sequence (Als) family, Eap1, Hwp1, Ywp1 and Integrin-like protein (Int1p) that bind to host ligands (collagen type I and IV, laminin, fibronectin, entactin and vitronectin) reviewed by Munro (2010). For example, Klotz and Smith (1991) performed initial experiments using type I collagen derived from bovine skin and demonstrated that _C. albicans_ adhered rapidly, with almost half the yeast cells adhering within 15 minutes after their addition to the type I collagen coated wells.

### 2.6.2.2 Morphologic transition and invasion

*C. albicans* under different conditions exhibits various morphological forms such as unicellular budding yeast cells, filamentous hyphae or pseudohyphae. This is also known as fungal dimorphism or fungal polymorphism (Sudbery et al. 2004; Gow et al. 2012).

*C. albicans* yeast cells are characterized by round to ellipsoid shaped cells. During initial colonization the mother yeast cell undergoes cytokinesis to produce a daughter cell that is smaller in size. Provided the environmental and host conditions are favorable, the yeast cells exhibit hyphae growth, that are long, narrow-like cells generally ≤ 2 microns wide with parallel sided walls and no obvious constriction at the site of septation-walls between cells within the hypha (Hedden & Buck 1980).
It has been suggested that *C. albicans* cells are able to sense the density of the surrounding *C. albicans* population by a quorum sensing mechanism that is based on the compound farnesol, which is secreted into the environment and inhibits hyphal formation (Hornby *et al.* 2001; Hogan 2006). It is believed that this may be due to down regulating cyclic AMP-protein kinase A complex signaling (Deveau *et al.* 2010).

It is generally believed that *C. albicans* hyphae are associated with host tissue invasion (Wachtler *et al.* 2012). *C. albicans* can use two distinct mechanisms to invade host tissues: induced endocytosis and active penetration (Zakikhany *et al.* 2007; Dalle *et al.* 2010). Active penetration is presumed to rely on a combination of physical pressure exerted by extending hyphae and secretion of hydrolytic enzymes.

Hyphae have been shown to invade 8% (w/v) agar which has an opposing resistant force of 0.1µN µm$^{-2}$ (Gow *et al.* 2002). *C. albicans* can invade agar when the yeast form is stab-inoculated into culture media containing 8% agar, where the cells grow spontaneously as hyphae penetrating the agar (Brush & Money 1999). This opposing pressure of resistance is not present in dentinal tubules of infected teeth which means hyphae may freely invade dentinal tubules, though the exact mechanisms underlying active penetration are still unknown.

The secretion of hydrolytic enzymes from the hyphal tip is suggested to aid in active penetration. The hyphal tip secretes enzymes that degrade proteins, lipids and other cellular components that may initiate or further facilitate hyphal invasion into solid substrates and tissues, presumably by liquefying or softening the substrate in front of the advancing hyphae tip (Hube & Naglik 2001).

Hydrolytic enzymes are classified into three groups: secreted aspartyl proteinases (Sap), phospholipases and lipases (Naglik & Hube 2010). *C. albicans* only possesses Sap of which 10 have now been identified, Sap1 through to Sap10 (Moran *et al.* 2004). A recent paper identified that Sap2 was able to digest human proteins found on mucosal surfaces such as extracellular matrix...
proteins and mucin (Naglik et al. 2003). The question remains, whether Sap1-10 plays a direct or indirect role in pathogenicity; aspects of fungal penetration, invasion and immune evasion are still largely unknown. However, the fact remains that collagen is the most abundant protein in the extracellular matrix (Di Lullo et al. 2002) which is also present in dentine and is a potential adhesion substrate.

Dentine is an amorphous mineralized tissue, consisting of seventy percent mineral hydroxylapatite, twenty percent organic material and ten percent water (Tencate 1998). The organic matrix of dentine consists of 90% fibrous collagen type I and the remainder consists of non-collagenous proteins, proteoglycans and lipid-containing components (Linde & Goldberg 1993).

2.6.2.3 Hyphal growth

The hyphal apex has plastic properties containing microfilaments (chitin and glucan) that become progressively more rigid through crystallization and cross-linking by covalent bonds. Hyphal growth occurs at the apex (tip), which involves the transportation of vesicles produced by the Golgi bodies to the apex. The vesicles provide new cell membrane material. These vesicles carry membrane bound enzymes such as chitin synthase in chitosomes and other vesicles carry material for secretion such as wall mannoproteins and extracellular enzymes (Gooday 1995). The vesicles accumulate in the Spitzenkorper (apical body) before being transported to the apical dome (apex tip). This process allows the hyphae to extend and move forward as described by a mathematical model (Bartnicki-Garcia et al. 1989). Microtubules are also present in the advancing hyphae tip, however their exact role is still not clear.

Brand and colleagues (2007), demonstrated that C. albicans hyphal tip growth and topographical orientation is regulated by a calcium-dependant mechanism. They proposed a model whereby the hyphal tip which comes into contact with a ridge causes the membrane to stretch (detected by Mid1p, a mechanosensor that activates calcium influx through Cch1p, a voltage-gated Ca\(^{2+}\) channel) allowing the influx of Ca\(^{2+}\) through the activated Mid1p. The area will have a localized
Ca\textsuperscript{2+} concentration that redirects cell polarity to a new growth axis in the direction of the Ca\textsuperscript{2+} influx (Brand et al. 2007). However, calcium channels have not been identified in \textit{C. albicans}. Therefore, based on high-affinity and low-affinity calcium uptake systems described, and partially characterized, in other species namely \textit{Saccharomyces cerevisiae}, the authors identified and deleted these homologs in \textit{C. albicans}. These homologs correspond to the voltage-gated Cch1p channel and the stretch-activated channel Mid1p (high-affinity) and Fig1p (low-affinity) calcium uptake systems (Brand et al. 2007). The deletion of these homologs was suggested to be associated with directing hyphal growth. The hyphae morphologic state is associated with invasion of various structures (Sherwood et al. 1992; Gow et al. 1994a).

\textit{2.6.2.4 \textit{C. albicans} biofilms}

Biofilms are highly structured communities of microorganisms that are surface associated, either attached to the surface or to one another within a self-produced and maintained extracellular matrix (Costerton et al. 1995). The advantages of biofilms to cells include providing protection to organisms from the surrounding environment, increased resistance to removal by physical, chemical or combination of the two and metabolic cooperation (Jabra-Rizk et al. 2004). Several materials have been used to demonstrate biofilm models. For example, latex material produces the best \textit{C. albicans} biofilm on synthetic materials followed by PVC and polyurethane, silicone (Hawser & Douglas 1994) and more recently on polymethylmethacrylate (PMMA) strips. It was shown that biofilm development was influenced by the inoculum size, adherence time, incubation time and exposure to nutrients (Chandra et al. 2001).

\textit{C. albicans} can directly attach to one another or to bacterial organisms that have already colonized the substrate (El-Azizi et al. 2004). For example, \textit{C. albicans} and \textit{Streptococci} appear synergistic whereby the bacteria provide adhesion sites and excrete lactate that may act as a source of carbon for yeast to grow (Jenkinson et al. 1990; Holmes et al. 1995) and a more recent study on \textit{Streptococcus gordonii} an oral microbe found on most oral cavity surfaces also
attaches to and interacts with \textit{C. albicans} to promote hyphae biofilm-formation. This relationship is also known as coaggregation (Silverman \textit{et al.} 2010).

\textit{C. albicans} biofilm development starts with yeast attachment followed by rapid growth and formation of micro-colonies. Hyphae are formed and deposition of the extracellular matrix material occurs and subsequent biofilm development follows. Hyphal growth may strengthen the entire structure and provide protection and adhesion sites for the budding yeast cells (Chandra \textit{et al.} 2001). This process can be divided into three phases based on microscopic analysis, which are: Early (0-11 hrs), intermediate (12-30 hrs) and maturation (38-72 hrs). Within the first few hours micro-colonies of budding yeasts are followed by pseudo-hyphae and true hyphae being present around 4-8 hrs. The intermediate phase involves the conjoining of micro-colonies by hyphal extensions, which is distinctly identified by the development of an opaque film covering the micro-colonies made up of non-cellular material. This opaque film appears cloudy due to the extracellular material composed of the cell-wall-like polysaccharides. Yeast cells make up the basal layer, and hyphae compose the structural framework (Ramage \textit{et al.} 2001). The maturation phase is where the quantity of the extracellular material increases until the communities formed are enclosed to form a mature biofilm (Chandra \textit{et al.} 2001). \textit{C. albicans} biofilms can range from between 50 and 350 \textmu m thick when viewed under confocal laser scanning microscopy (Mukherjee \textit{et al.} 2009). The role of yeast biofilms in pathogenesis of endodontic pulpal and periapical infections is still unknown.

\textit{2.6.2.5 Dentin colonization}

\textit{C. albicans} must first be present in the oral cavity followed by initial adherence to a surface for colonization to occur and infection to persist (Odds 1988). For example, \textit{C. albicans} and hyphal structures have been observed to have a strong affinity to dentine, enamel and cementum \textit{in vitro}. Hyphae were observed to be in close contact to surfaces, even when growing over the edge and penetration of cracks whenever present. This led the authors to believe that \textit{Candida} may be dentinophilic in nature (Sen \textit{et al.} 1995; Sen \textit{et al.} 1997a; Sen \textit{et al.} 1997b).
A further study showed that the presence of a smear layer increased the adhesion of *C. albicans* (Sen *et al.* 2003). They hypothesized that this may be attributed to the components of dentine such as collagen and calcium ions that is a source of nutrition and substrate for adhesion and growth.

In addition, an *in vitro* study demonstrated that only a small number of *C. albicans* hyphae penetrated dentinal tubules up to 60 µm (Waltimo *et al.* 2000). This may be due to the use of agents that remove the smear layer such as sodium hypochlorite (NaOCl) and ethylene diaminetetraacetic acid (EDTA) (Siqueira & Sen 2004).

### 2.7 Pulp and periapical disease

The major cause of pulp necrosis and periapical disease is the presence of bacteria in the tooth. Miller (1890) was the first to document the presence of several bacteria in necrotic pulps in teeth. It was not until over half a century later that Kakehashi *et al.* (1965) proved that bacteria cause pulp disease. Kakehashi and associates exposed the pulps of molar teeth in germ-free rats and normal rats with oral bacteria. They found that the pulps of the germ free rats remained healthy while the pulps in rats with bacteria had abscess formation and necrosis. Similar findings came from the work of Möller *et al.* (1981) when they intentionally infected pulp tissue in monkeys and compared this group to another with non-infected necrotic pulp tissue. It was shown that the latter did not induce inflammation in the apical tissues whereas the bacteria-infected pulp tissue showed inflammation and periapical radiolucencies. Fabricius *et al.* (1982) in a study involving monkeys, found no inflammatory reactions in devitalized non-infected teeth. These studies suggest that the exposed dental pulp remains healthy and has the capacity to heal provided there is no bacterial contamination.
2.8 Microbiota of infected pulps and root canals

In the past, the bacterial species that were associated with endodontics mostly demonstrated the presence of aerobic bacteria such as the *Streptococcus* species. This was the culture-dependant approach that only supported bacteria that tolerated aerobic conditions. It was not until studies utilized anaerobic culturing techniques that microbiota in necrotic dental pulps were reported as being predominately obligate, non-sporulating anaerobic bacteria such as *Fusobacterium*, *Porphyromonas* and *Prevotella* species (Sundqvist 1976; Möller et al. 1981; Shah & Collins 1990). Furthermore, the introduction and application of molecular biology techniques (such as nucleic acid techniques-PCR, DNA-DNA hybridization methods and DNA sequencing) to dentistry identified up to 700 species in the oral cavity (Munson et al. 2002; Sakamoto et al. 2006; Siqueira & Rocas 2010). However, these modern technological advances may be overly exaggerating the microorganisms implicated in infecting pulps and root canals. They detect both living and dead organisms and in minute numbers. A more reliable detection may be organisms that are cultivatable in the laboratory setting. Therefore caution must be taken when identifying the main organisms associated with pulp and periradicular disease. The ability to detect uncultivated or fastidious bacteria that may die during sampling, transportation or isolation procedures is an advantage in terms of identifying possible roles of these organisms in the pathophysiology of pulp and periapical disease (Munson et al. 2002). In contrast, the main downside of such sensitive detection techniques is that they cannot differentiate between live or dead bacteria, which may give rise to misinterpretation of their roles in pulp and periapical disease (Siqueira & Rocas 2010).

The endodontic bacteria commonly isolated from infected coronal and root dentine with varying degrees of frequency are: *S. mutans*, *S. intermedius*, *S. sobrinus*, *S. morbillorum*, *S. sanguinis*, *S. mitis*; *Peptostreptococcus anaerobius*, *P. parvulus*, *P. micros*; *Actinomyces israelii*, *A. naeslundii*, *A. odontolyticus*, *A. viscosus*; *Eubacterium alactolicum*, *E. aerofaciens*, *E. saburreum*; *Propionibacterium avidum*, *P. acnes*, *P. lymphophilum*, *P. propionicum*;
Lactobacillus casei, L. plantarum, L. minutus; Fusobacterium nucleatum, Bifidobacterium spp, Peptococcus spp, Clostridium spp, Porphyromonas spp, Prevotella spp and Veillonella spp (Love & Jenkinson 2002).

The bacterial species commonly found in root canals of teeth with no symptoms are similar to those found in infected coronal and root dentine and include gram-positive cocci (e.g. Streptococcus anginosus, S. sanguinis, S. mitis, S. mutans, Enterococcus faecalis, Peptostreptococcus micros and P. anaerobius), gram-negative cocci (e.g. Capnocytophaga ochracea, C.spitigena, Veillonella parvula, Campylobacter rectus, C.curvus), gram-positive rods (e.g Actinomyces israeli, A. naeslundii, Eubacterium alactolyticum, E. lentum, E. nodatum, E. timidum, Propionibacterium propionicum P. granulosum and Lactobacillus) and gram-negative rods (e.g. Fusobacterium nucleatum, Prevotella intermedia, P. melaninogenica, P. denticola, P .buccae, P. buccalis, P. oralis, Porphyromonas gingivalis, P. endodontalis and Bacteroides gracilis) (Sundqvist 1992a; Sundqvist 1992b; Sundqvist 1994; Le Goff et al. 1997).

The bacteria that are associated with in vivo pulp and root canal infection that are known to invade dentinal tubules in vitro are S. sanguis (Ørstavik & Haapasalo 1990; Perez et al. 1993), S. sobrinus, L. casei, A. naeslundii (Nagaoka et al. 1995a), S. mutans (Love et al. 1997), S. gordonii (Love 1996; 1997) and E. faecalis (Akpata & Blechman 1982; Haapasalo & Ørstavik 1987; Ørstavik & Haapasalo 1990; Love 2001). The latter three will be discussed as they have been selected for this research.

2.8.1 Bacterial invasion of dentine/tubules

Dentinal tubule invasion by bacteria occurs when dentine is exposed to the oral cavity and host defenses have been overwhelmed. This can be through carious lesions, through restoration margins, fractured restorations, periodontal procedures or pockets, enamel or dentine cracks and dental trauma.

There are several factors that influence tubule invasion these are collagen, adhesion receptors, tubule patency, nutrition and other substances in dentine.
Love and Jenkinson (2002) introduced a model demonstrating streptococcal bacteria adhering to unmineralized collagen type I through antigen I/II polypeptide adhesion (outlined in following paragraphs).

Dentinal tubule patency plays a major role in bacterial invasion. For example, bacteria invade dentine more freely when the smear layer is absent compared to when a smear layer is covering the tubule orifices (Olgart et al. 1974).

Nutrition is important for colonization and growth of bacteria, which can influence the penetration of bacteria in dentinal tubules. The majority of nutrition comes from within the root canal system (Siqueira 2008). Bacterial penetration from the exterior is initiated by saccharolytic bacteria consuming the carbohydrates allowing colonization on dentine (primary colonizers) and later giving way to assaccharolytic bacteria (secondary colonizers) which adhere to the initial colonizers.

2.8.2 *Streptococcus mutans*

*S. mutans* was first described by Kilian (1924). It is a facultative anaerobic, gram positive cocii-shaped bacterium. *S. mutans* is one of the normal oral microbiota that colonizes hard and soft tissues, and is identified as the primary cause of dental tooth decay. *S. mutans* metabolizes different kinds of carbohydrates for nutrition and growth, and it produces acidic by-products such as lactic acid that is known to demineralize the hydroxyapatite in enamel (Gibbons & van Houte 1975; Smith 2002). *S. mutans* has been suggested to be the most abundant bacteria in the oral cavity (Nyvad & Kilian 1990).

2.8.2.1 Pathogenicity

*S. mutans* plays a major role in pulp and periapical disease indirectly and possibly directly through the breakdown of the enamel protective layer to expose dentinal tubules to bacterial antigens (Levin 2003). The dentinal tubules communicate directly with the pulp and it is bacterial penetration through these channels that ultimately leads to pulpitis and periapical disease if not treated
early through preventive and conservative interventions (Hahn & Liewehr 2007). The pathogenicity of *S. mutans* is primarily by acid production “destroying” hydroxyapatite. In addition this bacterium has the ability to adhere to the host tissues, to evade host immune response in biofilms and to invade dentinal tubules (Love *et al.* 1997).

### 2.8.2.2 Adherence

*S. mutans* adheres to conditioned soft (oral mucosa, tongue, gingivae) and hard tissue (enamel, exposed dentine, root cementum) in the oral cavity (Nyvad & Kilian 1990). Cell surface proteins enable streptococcal bacteria to adhere to the salivary molecules that bind to tooth enamel (Lamont *et al.* 1991). Several have been identified—such as the antigen I/II family polypeptides, amylase-binding proteins, surface lectins, fimbrial adhesins, EP-GP binding protein, and glucan-binding proteins GBP74 and GBP59 (Love *et al.* 1997; Hubble *et al.* 2003).

The main receptor for *S. mutans* that permits adherence to the soft and hard tissues is a family of polypeptides identified as antigen I/II (Jenkinson & Demuth 1997; Jakubovics *et al.* 2005). The antigen I/II polypeptides are implicated in binding of *S. mutans in vitro* to a range of host receptors such as collagen type I, fibronectin, laminin and fibrinogen with varying affinity between *S. mutans* strains (Love *et al.* 1997; Beg *et al.* 2002). *S. mutans* NG8 adheres to collagen type I better when compared to the antigen I/II (spaP) mutant strain 834 (Love *et al.* 1997). However, *S. mutans* M51 adhered more to collagen type I compared to *S. mutans* NG8, which adhered more to fibronectin than collagen type I. *S. mutans* NG8 has about 40% more antigen I/II polypeptide on its surface compared to *S. mutans* M51, as determined by using reactivity of cells of the strains to anti-antigen I/II sera (Beg *et al.* 2002). The authors suggested that this may be because antigen I/II may only be one of several mechanisms that permit these cells to adhere. Therefore further studies need to be conducted to ascertain the differences in adherence properties.

A major host receptor for antigen I/II is the salivary agglutinin glycoprotein (SAG) which is secreted by the parotid gland and by secretory cells at other
mucosal sites (Leito et al. 2011). SAG, also known as gp340, is associated with the humoral innate immune response against *S. mutans* in vitro, whereby it promotes streptococcal aggregation and assists with the clearance of bacterial cells from the oral cavity through the normal swallowing reflexes. SAG may also be incorporated into the salivary pellicle on the tooth surface which may further act as a receptor for streptococcal adhesion and subsequently promote formation of the biofilm (Lamont et al. 1991). It has been suggested that there are at least two binding regions of antigen I/II in *S. mutans* that may be involved in SAG adhesion (Kelly et al. 1995). Following adherence to host tissues, the initial colonizers continue to multiply and encourage the formation of a biofilm.

### 2.8.2.3 Biofilm

One of the main virulence properties of *S. mutans* is the ability to form biofilms to which other bacteria may attach into co-existing habitats. A number of processes are involved in the development of a biofilm including positive or negative interaction between bacterial species (Yoshida & Kuramitsu 2002). Positive interactions may occur such as two species benefiting or one species benefits without affecting another species in the biofilm. For example, facultative bacterial metabolic reactions use up oxygen, creating an environment that supports anaerobic bacteria. In comparison, negative interactions limit a bacterial population within a biofilm, when two species compete for the same resource or when one species produces a substance that may inhibit the other (Siqueira 2008).

Biofilms, once established, protect the bacteria from antimicrobials (administered locally) and lead to an increased tolerance to the host’s defense system (Leid 2009; Marsh et al. 2011; Elias & Banin 2012). For example, *S. mutans* survives exposure to chlorhexidine, hydrogen peroxide, erythromycin and zinc chloride more successfully when present in a mixed biofilm than as a mono-species biofilm (Kara et al. 2006).

There are several factors which enable bacteria biofilms to be more resistant to host defenses and antimicrobial agents which are, limited penetration of
leukocytes and their products into the biofilm, quorum sensing amongst bacterial species, genetic switching that increases drug resistance of biofilm bacteria (Leid 2009).

2.8.2.4 Dentine tubule invasion

Love and Jenkinson (2002) reviewed the literature on invasion of dentinal tubules by oral bacteria and highlighted that streptococci were associated with in vivo dentine caries and invasion of coronal dentinal tubules. They are also primary colonizers of root canal infection and subsequently invade root dentinal (radicular) tubules in vitro. An earlier paper by the same authors showed that the streptococcal cell-surface antigen I/II polypeptides were associated with determining the ability of S. mutans to invade human root dentinal tubules, where wild-type S. mutans NG8 cells invaded dentinal tubules whereas the mutant strain lacking in the polypeptide I/II did not (Love et al. 1997).

However, there is considerable variation in the degree of penetration amongst the subspecies of S. mutans and the type of teeth used (e.g. human versus animal teeth). For example, an immunofluorescent antibody technique demonstrated that S. mutans invades dentine in teeth with open root canal walls in vitro. The distance of invasion from the root canal walls ranged from an average of 493-524 µm to a maximum of 1050-1150 µm. When they compared this to invasion revealed by Gram’s stain, it averaged 598 µm and reached a maximum of 1250 µm. The difference in distance of invasion revealed by these two methods was statistically significant, which suggests the more specific the technique, the more it will reveal the full extent of invasion (Kouchi et al. 1980). A later study utilizing SEM to investigate S. mutans colonization and invasion of sterile dentine blocks, found that S. mutans occasionally invaded the dentinal tubules and only to a depth of 5 µm. In addition, in the dentinal tubules which S. mutans invaded, a rapid destruction of the peritubular dentin sheath occurred. This supports the idea that acid by-products of S. mutans diffuse into the dentinal tubules and cause tissue damage in the deeper parts of dentine (Adriaens et al. 1987).
2.8.3  *Streptococcus gordonii*

*S. gordonii* is a common oral microbe which colonizes most human oral sites (Frandsen et al. 1991; Socransky et al. 1998). *S. gordonii* is a gram-positive non-motile coccus that grows in pairs or chains. They are one of the initial colonizers in the development of dental biofilms (Rosan & Lamont 2000), and are generally nonpathogenic commensal streptococci. Dental biofilms eventually lead to periodontal disease and dental cavities suggesting that species like *S. gordonii* indirectly causes pulp and periapical disease if no preventive or conservative treatment is provided.

*S. gordonii* cells produce cell surface associated polypeptide proteins that bind to host proteins or cells. These include fibronectin-binding proteins (McNab et al. 1996; Christie et al. 2002; Jakubovics et al. 2009), amylase-binding protein (Rogers et al. 2001), haemagglutinin (Takahashi et al. 2002) and platelet-binding protein (Bensing & Sullam 2002). Similar to *S. mutans*, *S. gordonii* has receptors for SAG allowing them to bind to the acquired pellicle containing SAG on the tooth surface. These are the cell wall-anchored polypeptide adhesins SspA and SspB, which belong to the antigen I/II family of polypeptides produced by most species of oral Streptococci (Jenkinson & Demuth 1997). Similarly Love et al. (2000) showed that *S. gordonii* mutants lacking the SspA and SspB proteins had deficient binding to collagen (a substrate of dentine) and reduced invasion of dentinal tubules. *S. gordonii* then acts as a binding site for pathogenic oral bacteria through a process called coaggregation. Therefore, the role *S. gordonii* has in the pathogenicity of pulp and periapical disease is probably as an early colonizer establishing a mono-species biofilm to which other bacteria may adhere.

2.8.3.1  Dentinal tubule invasion

Invasion of dentine by *S. gordonii* is influenced by the presence of a smear layer. Histological sections revealed *S. gordonii* penetrating all dentine coronal samples without a smear layer. In comparison, nine out of ten smeared samples exhibited no penetration by *S. gordonii*. This suggests that the dentinal smear
layer inhibits dentinal tubule invasion by bacteria. In addition, *S. gordonii* has been shown to invade dentinal tubules *in vitro* where they can penetrate up to 200 µm in the mid-root region and up to a maximum of 60 µm in the apical region (Love *et al.* 1996c).

A further study compared the growth of *S. gordonii* DL-1 on BHY medium containing collagen versus BHY medium without collagen. *S. gordonii* grew in short chains when grown on BHY medium, but formed long intertwined chains of cells in BHY medium containing 0.2mg of acid-soluble collagen/ml (Love *et al.* 1997). In this study the *S. gordonii* mutant strains lacking the cell surface protein SspA and SspB (which are known adhesins associated with specific binding of cells to a variety of host and bacterial receptors) did not invade dentinal tubules whereas with the wild type, significant invasion was evident when viewed under light microscopy. This suggests that collagen guides the ability of *S. gordonii* to form long chains in dentinal tubules, contributing to invasion which may be thigmotropic in nature.

**2.8.4 Enterococcus faecalis**

*E. faecalis* was first identified as a *Streptococcus* D bacterium (*Streptococcus faecalis*) because it contained a cell wall carbohydrate that was characteristic of *Streptococcus* D. However, post-1980s, DNA–DNA and DNA–RNA hybridization studies demonstrated a more distant relationship with the streptococci species (Portenier *et al.* 2003). *E. faecalis* is a gram-positive, spherical, facultative anaerobic bacterium normally found in the gastrointestinal tract. It appears singly, in pairs, or in short chains (Ryan & Ray 2004).

*E. faecalis* can cause an array or infections in humans such as endocarditis and bactearemia, urinary tract infections, and meningitis (Murray 1990). In the oral cavity, it is usually associated with teeth that have persisting periapical lesions following root canal treatment (Molander *et al.* 1998). In this situation these root canal-treated teeth are about nine times more likely to harbor *E. faecalis* than cases of primary infections (Rocas *et al.* 2004; Sedgley *et al.* 2006). This suggests that *E. faecalis* either lies dormant and is revived when
certain conditions favor it (such as availability of nutrients), or it may have entered the root canal system during or between visits or following completion of root canal treatment (Sundqvist 1992b; Sundqvist & Figdor 2003). The pathogenicity of *E. faecalis* in persistent periapical lesions and its implication in failed endodontic treatment (Sedgley *et al.* 2006; Williams *et al.* 2006) may be determined by a number of virulent factors including adherence to host cells and extracellular matrix, dentinal tubule invasion and antimicrobial resistance (Portenier *et al.* 2003). The first two virulence factors will be outlined in regards to this particular research.

### 2.8.4.1 Adherence

Adherence of *E. faecalis* to dentine is an important step in the invasion of dentinal tubules. *E. faecalis* has cell wall surface proteins (aggregation substance (AS), enterococcus surface protein (Esp) and collagen-binding protein (Ace) and proteases) that may enable the cells to adhere to dentine (Hubble *et al.* 2003).

AS is a pheromone-responsive, plasmid-encoded bacterial adhesin that mediates and facilitates the contact between the donor and recipient bacteria (Kayaoglu & Ørstavik 2004). AS is proteinaceous and appears as a hair-like structure on the cell surface, and it is incorporated into the older parts of the cell wall (Wanner *et al.* 1989). AS has been found to mediate binding to collagen type I (the main organic constituent of dentine), with different binding affinity amongst *E. faecalis* strains (Rodzinski *et al.* 2001).

The enterococcal geneEsp encodes the surface protein Esp which is a large chromosome-encoded surface protein containing multiple repeat motifs. The role Esp plays in virulence is still unclear, however it has been shown to participate in host interactions. A recent study demonstrated that in the presence of Esp the adherence and biofilm formation to surfaces were increased (Toledo-Arana *et al.* 2001).
Rich et al. (1999) identified a putative proteinaceous adhesin of *E. faecalis*; known as Ace, the collagen-binding MSCRAMM (microbial surface component recognizing adhesive matrix molecules). An *ex vivo* study demonstrated that Ace and serine protease (secreted enzyme) may aid *E. faecalis* binding to dentine (Hubble et al. 2003). In addition, the adherence of *E. faecalis* to dentine increases after starvation and when dentine is coated with saliva (George & Kishen 2007).

Other factors may affect the adhesion of *E. faecalis* to collagen. For example, *E. faecalis* adheres best to collagen type I at a pH of 8.0 to 8.5 (Kayaoglu et al. 2005). These properties are likely to be important in colonization and invasion of dentine.

### 2.8.4.2 Dentinal tubule invasion

*E. faecalis* invades dentinal tubules *in vitro* (Akpata & Blechman 1982; Haapasalo & Ørstavik 1987; Peters et al. 2000; Love 2001; Chivatxaranukul et al. 2008) and is also implicated in failed endodontic treatment (Molander et al. 1998; Sundqvist et al. 1998).

Love (2001) showed that the ability of *E. faecalis* to invade dentine and adhere to collagen type I was maintained in the presence of human serum, although at a reduced rate. In comparison, human serum affected and interfered with *S. mutans* and *S. gordonii* dentine invasion. In addition, *E. faecalis* invasion of dentinal tubules was inhibited by acid soluble collagen. He proposed that these properties allowed *E. faecalis* to colonize and invade dentinal tubules in a stressed environment and may account, in conjunction with other factors, for its ability to remain viable in a filled root canal and sustain a periapical lesion.

A later *in vitro* study, confirmed that collagen type I at a high concentration inhibited and displaced *E. faecalis* binding to dentine, that Ace mediates *E. faecalis* adherence to dentine and that anti-Ace IgG significantly inhibited attachment to dentine (Kowalski et al. 2006). This suggests that *E. faecalis* adherence and subsequent invasion of the dentinal tubules is dependent on the
binding of host un-mineralised collagen type I by cell surface proteins on the invading organism.

The depth of invasion in *in vitro* and *ex vivo* studies varies according to the conditions under which the studies were done. The depth of *E. faecalis* invasion ranged from 400 µm (Haapasalo & Ørstavik 1987) after one day to 1000 µm after three weeks incubation under ideal conditions. Another study demonstrated that after 21 days of incubation, *E. faecalis* invaded up to 1433 µm in a rich nutrient-aerobic condition (George *et al.* 2005). However, another study suggested that invasion is time dependant in which *E. faecalis* invaded the root canal dentine only after two weeks of incubation. After three weeks of incubation the bacteria were seen to invade almost the full length of dentine under anaerobic conditions (Akpata & Blechman 1982). In addition, *E. faecalis* invasion was less in a nutrient-deprived environment-620 µm (with aerobic conditions) and 650 µm (with anaerobic conditions).

*E. faecalis* has the ability to survive and withstand long periods of starvation, and subsequently recover in the presence of serum (Figdor *et al.* 2003). This was supported by an *ex vivo* study where *E. faecalis* maintained viability after 12 months (Sedgley *et al.* 2005). There is a possibility that bacteria entombed at the time of obturation may result in endodontic treatment failures, when there is a compromise in the overall seal allowing saliva components and serum back into the canals to provide a source of nutrition for *E. faecalis* (Rocas *et al.* 2004).

### 2.8.4.3 Biofilm

*E. faecalis* has the ability to form biofilms within a root canal (Nair *et al.* 1990; Wang *et al.* 2011). It can modify these biofilms into a biomineralised calculus-like structure (Kishen *et al.* 2006) that may provide added protection from the host immune system. This structure may be from the accumulation of calcium (George *et al.* 2005). *E. faecalis* has been shown to form single-species biofilms, suggesting it has an antagonistic relationship with other root canal inhabitants (Sundqvist *et al.* 1998). This bacterium can also form biofilms with other selected bacteria such as *Fusobacterium nucleatum* (Johnson *et al.* 2006), *S.*
**mutans** (Deng *et al.* 2009) and other gram-positive facultative anaerobes (Takemura *et al.* 2004; Al-Ahmad *et al.* 2009). In addition, these biofilms have also been suggested to be associated with *in vivo* medicated root canals. Distel *et al.* (2002) showed using SEM that *E. faecalis* biofilm can survive in canals medicated with calcium hydroxide paste even after 77 days.

### 2.9 Polymethylmethacrylate plastic discs

Polymethylmethacrylate (PMMA) is a transparent thermoplastic material, sometimes called acrylic. Chemically, it is the synthetic polymer of methylmethacrylate. This material was developed in 1928 and first introduced onto the market in 1933 by a German chemist who patented and registered this material under the trademark Plexiglass™.

PMMA is widely used in the medical and dental professions because this material has good biocompatibility with human tissues. For example, in the medical field, PMMA is used in replacement intraocular lenses, and as a bone cement to affix implants and to remodel lost bone following orthopaedic surgery. In the dental field, PMMA is used to make dentures and dental materials such as composite resins with PMMA being the main component.

PMMA’s advantage is that it allows extremely high resolution (nanoscale) patterns to be made. For example, smooth PMMA surfaces and nanostructured PMMA surfaces can be processed by vacuum ultraviolet irradiation and oxygen radio-frequency plasma (Lapshin *et al.* 2010).

The ability to form grooves of a specific size and depth on PMMA surfaces may be particularly useful for *in vitro* studies to simulate dentinal tubules. In addition, proteins may be used to coat the PMMA substrate to replicate the organic portion of dentinal tubules (Kang *et al.* 1993; Alaerts *et al.* 2001). Collagen type I is the main component in dentine (Linde & Goldberg 1993). Therefore when it is adsorbed onto grooves that simulate dentinal tubules it will permit *in vitro* studies to observe the behaviour of oral bacteria and devise
possible strategies (e.g. development of bio-active peptides to eliminate or inhibit growth into or along the dentinal tubules).

2.10 Glycine-Proline-Alanine

2.10.1 Collagen type I

As mentioned earlier, collagen is the most abundant protein in the extracellular matrix (Di Lullo et al. 2002) which is also present in dentine and is a potential adhesion substrate.

Collagen type I is made up of rod-like collagen molecules. Each consisting of three α chains, comprising 1000 amino acid residues arranged in a triple helical structure. This helical structure is not collagen. Collagen is synthesized in odontoblasts as pro-collagen and converted by membrane bound enzymes known as collagen peptidases into tropocollagen in the pre-dentine layer. Formed collagen fibrils are stabilized by subsequent formation of covalent cross-links between tropocollagen molecules to form polymers of tropocollagen known as a collagen fibril (Linde & Goldberg 1993). The cross-linking creates gaps (hole zones) and overlapping molecules (overlap zones), creating a cross-striation appearance when viewed under an electron microscope (Eyre 1987).

Lin et al. (1993) demonstrated similar findings where they described collagen fibrils in human dentine coursing longitudinally to merge into a transversely interwoven fine fibrillar network. They also examined collagen fibrils by high-resolution field-emission scanning electron microscope (FESEM), revealing closely packed collagen fibrils with diameters of 30 to 50 nm.

2.10.2 Amino acids associated with collagen type I

Amino acids are the molecules which make up proteins. The most common amino acid sequences of collagen are Glycine-Proline-X and Glycine-X-Hydroxyproline, where X is any amino acid other than glycine, proline or hydroxyline. Szpak (2011) analysed the amino acid composition of mammalian
skin and showed that the third most common amino acid is alanine followed closely by hydroxyproline. However, the composition in hard tissue is considerably different. For example, Armstrong (1961) analysed the amino acid residue per 100 g dry dentine preparation in sound dentine, and demonstrated that the most common were glycine, proline, hydroxyproline, glutamic then alanine. In comparison, in carious dentine the most to least common were glycine, glutamic, alanine, proline and hydroxyproline. The other detected amino acids in lesser amounts were aspartic, threonine, serine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, hydroxylsine, histidine, lysine and arginine.
2.11  Aim

The aim of this research was to determine whether *E. faecalis* JH2-2, *S. mutans* NG8 or *S. gordonii* DL-1 exhibit the characteristics of contact guidance along polymethylmethacrylate (PMMA) discs.

2.12  Objectives

i. The primary objective is to determine whether bacteria use contact guidance to navigate along simulated dentinal tubules.

ii. To assess bacterial growth on PMMA discs.

iii. To identify which growth conditions support bacterial growth on PMMA grooved discs (submerged in a suspension of BHY broth or the firm surface of BHY agar, after one day, two days, five days and seven days.).

iv. To observe whether collagen type I encourages adhesion and colonization of bacteria to the discs.

v. To confirm the thigmotropic properties of *C. albicans* (positive control).

vi. To see whether the tripeptide Glycine-Proline-Alanine affects growth on PMMA discs.

2.13  Hypotheses

1. *C. albicans* ATCC 10261 will exhibit contact guidance (thigmotropism) along simulated dentinal tubules.

2. Since bacteria are prokaryotes they will not exhibit contact guidance.
3 RATIONALE FOR EXPERIMENT

Mechanisms that microorganisms use to invade dentinal tubules have not been fully determined. *E. faecalis JH2-2*, *S. mutans NG8* and *S. gordonii DL-1* were selected to investigate one potential mechanism thigmotropism because these organisms are commonly found in endodontic infections. *C. albicans* has been identified in dentinal tubules and has also been reported to exhibit the characteristics of thigmotropism *in vitro* on several surface substrates. Therefore, *C. albicans* was used as a control organism in this *in vitro* study. A pilot study was undertaken as a summer research project by Nadell Ibriahim (RC Tonkin Summer Studentship 2007-2008), at the School of Dentistry, University of Otaga, to investigate contact guidance by bacteria. A major breakthrough since then has been the manufacturing of plastic discs with grooves 2 μm wide compared to the larger 10 μm grooves used by Ibriahim (2007-2008). Therefore it is now possible to test various combinations of grooves and spacing.

3.1 Significance of research

*C. albicans* exhibits the characteristic of contact guidance along grooves in membranes (Sherwood *et al.* 1992; Gow *et al.* 1994b; Sen *et al.* 1997a). This phenomenon has not been investigated in the field of dentistry using bacteria. It is generally accepted that bacteria are the main cause of pulp and periapical disease, therefore it is important to determine whether bacteria exhibit the same contact-guidance. This may augment what is now known about bacteria and how they may propagate through dentinal tubules of human teeth. Furthermore, data collected may assist in further clinical research to determine and identify possible peptides or molecules that may potentially prevent bacterial invasion into dentinal tubules before they can cause irreversible damage to the pulp and periapical tissues.
4 METHODS

Ethical approval was not required because humans or animals were not involved in this research. The experiments were conducted on grooved PMMA discs that simulated some characteristics of dentinal tubules. The bacteria selected for this research were Streptococcus mutans NG8, Streptococcus gordonii DL-1, Enterococcus faecalis JH2-2 and the yeast Candida albicans ATCC 10261. The experiments were refined through a series of pilot trials. The pilot trials were performed to determine the ideal conditions required for these organisms to grow on PMMA.

4.1 Preparation of polymethylmethacrylate discs

Polymethylmethacrylate discs of approximately 15 mm in diameter and 0.5 mm thick were made from PMMA. A grooved surface of 5 x 5 mm was made from UV photo-definable Su8 epoxy using standard lithography (Fig 4.1.a) to decrease the bonding temperature of the PMMA discs and increase the reliability of the bonding process (Svoboda et al. 2010). Briefly the process consisted of laying out a L-Edit CAD plotting on a plastic reticule using a pattern generator, that was then reduced in size 10-fold onto a chrome mask with a step-and-repeat camera. This pattern was then transferred to the Su8 epoxy on a mask aligner. Adhesion of the pattern was improved by both chemical and oxygen-plasma cleaning the plastic substrate. Following this, the Su8 epoxy was spun on to the substrate at 3000 rpm and pre-baked at 95°C. Unexposed epoxy was then removed using a chemical developer to leave a 3D wall of Su8 epoxy and channels on the discs (Bubendorfer et al. 2007). Four groups of PMMA discs were produced with grooves (Fig 4.1.b): Group A. 2 µm x 2 µm x 2 µm (width x depth x spacing); Group B. 2 µm x 2 µm x 4 µm with either; Group C. 4 µm x 4 µm x 2 µm and D. 4 µm x 4 µm x 4 µm. Groups A and B were used for experiments with bacteria, and groups C and D were for experiments with fungi, since they are larger than bacteria. A fifth group (E) served as a control and
consisted of blank PMMA discs (omnicoated) with no grooves. The discs were manufactured by Industrial Research Limited, Wellington, NZ.

Figure 4.1.a. Diagram of a PMMA disc showing the microgrooved surface (square with fine lines) (L = 15 mm, T = 0.5 mm).

Figure 4.1.b. Representation of microgroove dimensions (cross-sectional view).
The discs were broken into four equal parts as the initial size did not fit into the SEM. This was done before disinfection with sodium hypochlorite (5.25%) (NaOCl).

4.2 Microorganisms

4.2.1 Preparation of bacteria

The bacteria, *Streptococcus mutans* NG8, *Streptococcus gordonii* DL-1, *Enterococcus faecalis* JH2-2, were sourced from the microbiological laboratory (School of Dentistry, University of Otago, NZ). Bacteria were suspended in 15% (vol/vol) glycerol stock in Brain Heart Yeast (BHY) in sealed tubes and stored in at -80°C. A small amount was removed aseptically while still frozen and the tube was placed back into the freezer. The sample was streaked on to Tryptic soy broth and yeast (TSBY) agar plates (Trypticase Soy Broth 30 g/L Becton Dickinson Microbiology Systems, Cockeysville, MD, USA; Bactopeptone 5 gL ; Difco, Fort Richard; Yeast Extract 5 g/L, Difco Laboratories, Detroit, MI, USA and bacteriological agar 15 g/L ; Germantown, Danisco NZ Ltd ) using an aseptic technique (metal loop heated over Bunsen flame). The agar plates were placed into an anaerobic incubator at 37°C for 24 h.

A single colony was re-suspended into 10 ml brain heart infusion broth (BHY) containing BHI (37 g/L ; Difco Laboratories, Detroit, MI, USA) supplemented with yeast extract (5 g/L ; Difco) and incubated at 37°C in closed 20 ml glass universal tubes without shaking for 24 h. The broth was harvested by centrifugation (11.5 G for 8 min) and the white pellet was re-suspended in 5 ml BHY broth containing 15% (v/v) glycerol. This was mixed on a vortex electronic mixer. The bacteria stock was stored in 1.5 ml sterile tubes (Raylab, Glendene, Auckland, NZ) containing 1ml glycerol stock and stored at -80°C until required.

Cross matching of *S. mutans* NG8, *S. gordonii* DL-1 and *E. faecalis* JH2-2 was carried out to identify if the organisms used were of pure culture. This was done

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by incubating a colony of *S. mutans* NG8, *S. gordonii* DL-1 and *E. faecalis* JH2-2 on blood agar (sheep blood, Venous Supplies; Otago, NZ) under anaerobic conditions for 24 h at 37°C. Microbiology staff personnel confirmed the organisms used were of pure culture before proceeding with preparing stock cultures of each organism.

In preparation for the experiments, the glycerol stock was thawed and mixed on a vortex, and suspensions of *E. faecalis* JH2-2, *S. gordonii* DL1 (Challis) and *S. mutans* NG8 were adjusted to an optical density of 0.5 (1.0 x 10⁶ colony forming units (cfu)/ml) in BHY before being used to colonize PMMA discs.

4.2.2 Preparation of *Candida albicans*

The most commonly used *C. albicans* strain in the Molecular Microbiology Laboratory (Department of Oral Sciences, University of Otago) is *C. albicans* ATCC 10261 (American Type Culture Collection, Manassas, VA). *C. albicans* ATCC 10261 was suspended in 15% (vol/vol) glycerol stock in containing YPD in sealed tubes and stored in an upright -80°C freezer. A small amount was removed aseptically (steel loop and flame technique) and the sample was streaked onto yeast extract peptone dextrose YEPD agar plates (Yeast extract (10 g/L; Difco), Bactopeptone (20 g/L ; Difco), Glucose (20 g/L ; Difco Laboratories) and bacteriological agar 20 g/L (Germantown, Danisco, NZ Ltd) using an aseptic technique (metal loop heated over bunsen flame). The agar plates were placed into an aerobic incubator at 30°C for 24 h.

A single colony was re-suspended into 10 ml YPD broth [Yeast extract (10 g/L ; Difco Laboratories), Bactopeptone (20 g/L ; Difco Laboratories) and Glucose (20 g/L ; Difco Laboratories)] and incubated at 30°C in 20 ml glass universal tubes with shaking for 24 h. The broth was harvested by centrifugation (11.5 g for 8 min) and the pellet was re-suspended in 5 ml YPD broth containing 15% (v/v) glycerol. This was again mixed in a vortex electronic mixer. The *C. albicans* ATCC 10261 stock was stored in 1.5 ml sterile tubes (Raylab, Glendene, Auckland, NZ) containing 1 ml glycerol stock stored at -80°C until required.
In preparation for the experiments, the glycerol stock of *C. albicans* ATCC 10261 was thawed and mixed on a vortex. The suspension was adjusted to an optical density of 0.5 (1.0 x 10^6 cfu/ml) in YPD before being used in microbial colonization of PMMA discs.

### 4.3 Microbial colonisation of PMMA discs

#### 4.3.1 Preparation of discs

PMMA discs were disinfected before inoculation by soaking them in NaOCl for 30 sec they were then rinsed in distilled water for 30 sec, and then soaked in distilled water for 5 min to ensure the NaOCl was washed off.

#### 4.3.2 Determination of growth characteristics

The first part of the experiment investigated the ideal conditions and length of incubation for growth of *S. mutans* NG8 and *C. albicans* ATCC 10261 on the PMMA discs. Using the aseptic techniques, cells of *S. mutans* NG8 were streaked onto BHY agar and incubated at 37°C under anaerobic conditions without shaking. Similarly *C. albicans* ATCC 10261 cells were streaked onto YPD agar and incubated at 30°C under aerobic conditions without shaking. Before the agar plates were placed into the respective incubators, PMMA discs were placed on the agar medium and subsequently removed after 1 d, 2 d, 5 d and 7 d incubation with the microorganisms.

In the second part of the first investigation one, discs were placed into 2 ml sterile wells and inoculated with cell suspensions of *S. mutans* NG8 and *C. albicans* ATCC 10261 with optical density of 0.5 (1.0 x 10^6 cfu/ml). The *S. mutans* NG8 were suspended in BHY and incubated anaerobically for the various time frames stated above, whereas *C. albicans* were suspended in Glucose Salts Biotin (GSB) [(1g/L (NH₄)₂SO₄ (7.57 mM), 2g/L KH₂PO₄ (14.7 mM), 50mg/L MgSO₄.7H₂O (0.2 mM), 50mg/L CaCl₂.2H₂O (0.34 mM), 0.05 mg/L biotin, and 20g/L glucose (111 mM)] and 20% bovine foetal serum, and
incubated at 30°C under aerobic conditions for 3 h, 4.5 h and 6 h. The *C. albicans* ATCC 10261 samples were viewed under light microscopy (40x) to observe which time period gave the optimum growth of hyphae on the tested substrate (PMMA grooved disc). The *S. mutans* NG8 samples were prepared for SEM evaluation because the cells were too small to be viewed under light microscopy. PMMA discs were inoculated with test organisms as above. PMMA discs with dimensions 4 µm x 4 µm x 2 µm Width x Depth x Spacing (WDS), 4 µm x 4 µm x 4 µm (WDS) and a blank disc were used for *C. albicans* ATCC 10261 experiments. *S. mutans* NG8, *S. gordonii* DL-1 and *E. faecalis* JH2-2 were inoculated on 2 µm x 2 µm x 2 µm (WDS), 2 µm x 2 µm x 4 µm (WDS), 4 µm x 2 µm x 2µ m (WDS) and blank discs. Before SEM processing, the discs were rinsed with 1ml phosphate buffered saline (PBS)-Invitrogen (Life Technologies) for the bacteria experiments and GSB for *Candida* to remove any unbound cells. Each experiment was completed in triplicate. The discs were transported in a sealed container for SEM processing.

4.3.3 Microbial growth on collagen coated PMMA discs

PMMA discs were coated with collagen type I, which is found in dentinal tubules and is a known adhesion receptor for bacteria and *Candida*.

Collagen (Type I Rats tail C7661 Sigma-Aldrich, Sydney, Australia) was dissolved in 0.1M acetic acid to obtain a 1mg/ml [0.1% (w/v)] collagen solution. This was stirred at room temperature for approximately 3 h or until the collagen completely dissolved. The collagen solution was transferred to a sterile 5 ml screw capped glass bottle containing a carefully placed layer of chloroform (10% v/v of collagen solution) on the bottom. The bottle was left in a static position overnight at 4°C and the top layer containing the collagen solution was then removed aseptically. This technique was used to sterilise the collagen as other techniques, such as membrane filtration, would lead to substantial protein loss. The discs were coated with 6-10 µg collagen/cm² of disc. The collagen was left to bind overnight at 4°C. Excess fluid was then removed from the coated surface using 1 ml PBS and allowed to dry overnight. The discs were rinsed with
1ml PBS before introducing the cells and medium as described above. The bacteria were incubated for 2 days anaerobically at 37°C and *C. albicans* ATCC 10261 incubated for 3 hours at 30°C.

4.3.4 Microbial growth on collagen coated PMMA discs in Glycine-Proline-Alanine

In order to investigate the potential role of a proline-rich peptide on cell adhesion and growth, gly-pro-alá (HanDan YuShuo Chemical Exp&Imp Co., Ltd; Republic. China) was diluted according to the manufacturer’s instructions (1mg/3ml) in the broth of bacteria and *Candida* followed by pipetting into the wells containing the PMMA discs. The bacteria were incubated for 2 days anaerobically at 37°C and *C. albicans* ATCC 10261 incubated for 3 hours at 30°C.

4.4 Processing protocol for conventional SEM

Samples were first fixed in 2.5% glutaraldehyde made up in 0.1M cacodylate buffer for 2 hours, followed by washing in 0.1M cacodylate buffer 3 x 5 minutes to remove excess fixer. Secondary fixation of the discs in 1% osmium tetroxide made up in 0.1 M cacodylate buffer for 30 minutes, then washed (3 x 5 min) in 0.1M cacodylate buffer. The discs were dehydrated in 100% ethanol for 30 minutes. The discs were dried in a Bal-Tec CPD-030 critical point dryer (Bal-Tec AG, Balzers, Liechtenstein). Samples were mounted on aluminium stubs using double-sided carbon tape and coated with 10 nm gold palladium in a Emitech K575X Peltier-cooled high resolution sputter coater (EM Technologies Ltd, Kent, England). Samples were viewed in a JEOL JSM-6700F field emission scanning electron microscope (JEOL Ltd, Tokyo, Japan).

4.5 Processing protocol for Cryo-SEM

Samples were mounted on an Alto brass holder using cryo glue, then plunge frozen in liquid nitrogen slush. The holder was then transferred to a Gatan Alto 2500 cryo preparation chamber/cryo stage (Gatan Inc, Pleasanton, California,
USA). The chamber was kept at a constant temperature of -135°C. The samples were sublimed (-100°C for approximately 2 min) to remove a small amount of ‘frost’ from the top of the sample. The samples were then coated with 5 nm of gold palladium before being introduced to the chamber of the JEOL JSM-6700F field emission SEM (JEOL Ltd, Tokyo, Japan).

4.6 Defining contact guidance

Contact guidance was based on cell growth characteristics between non-grooved and grooved surfaces. This was when cells met either of the following criteria: (1) Chain growth and hyphae growing along an edge, (2) cells or hyphae encountering an edge and reorienting growth to continue growth along the edge, (3) cells or hyphae growing along the floor of a groove against the wall, (4) cells or hyphae growing along a groove encountering a wall, re-orientating and continue growing along the wall, and (5) cells or hyphae growing over a wall and ridge whilst staying in close contact with the substrate. These criteria have been modified from earlier studies on contact guidance using fungi (Sherwood et al. 1992; Gow et al. 1994a).
5 RESULTS

5.1 Microbial growth on agar and in broth

After day 1 *S. mutans* NG8 and *C. albicans* ATCC 10261, formed a very fine film over the agar with a streaky appearance. However, *S. mutans* NG8 and *C. albicans* ATCC 10261 showed increasing growth over 2, 5 and 7 days; a thick lawn was observed for both organisms. However, with experiments involving PMMA discs placed on the surface of the agar, no cells grew on blank or grooved PMMA discs when viewed with SEM after incubation at the respective time frames.

For *S. mutans* NG8, the bacteria presented as single cells, paired cells or as chains on smooth (Fig 5.1.a) and grooved (Fig 5.1.b) PMMA discs after two days incubation at 37°C. Similarly *C. albicans* ATCC 10261 demonstrated the optimum hyphal growth on both blank (Fig 5.1.c) and grooved (Fig 5.1.d) PMMA discs when viewed with 40x light microscopy (Olympus microscope LH50, Optical co Ltd, Japan), when grown in GSB containing 20% serum (foetal bovine serum) for 3 hours. The hyphae extended up to and more than 20 µm in length from the yeast cell (parent cell that has a known diameter of 4 µm). An earlier test demonstrated no hyphal growth when *C. albicans* ATCC 10261 was grown in YPD broth (results not shown).

The ideal condition of growth and length of incubation for the bacteria was in broth at 37°C for two days and *C. albicans* ATCC 10261 was in GSB containing 20% serum (foetal bovine) for 3 hours incubated at 30°C. These were the ideal conditions for both organisms; to assess thigmotropic properties on smooth and grooved discs. The bacteria growth was not ideal after one day because there were not many cells adhering to the discs after processing for SEM. Most of the cells were either single or in pairs. No chaining growth was observed. In contrast, at 5 and 7 days there was too much growth covering the discs. *C. albicans* ATCC 10261 hyphae lengths were similar after 3, 4.5 and 6 hours.
Figure 5.1.a. Representative SEM of *S. mutans* NG8 on a smooth PMMA disc. Cells grew as single or paired cells or as chains.

Figure 5.1.b. Representative SEM of *S. mutans* NG8 incubated on 4 µm x 4 µm x 2 µm grooves. Paired cell and chain growth are typically represented.
Figure 5.1.c. Light microscopy (40 x magnification) of *C. albicans* ATCC 10261 incubated on smooth PMMA disc. Note randomly directed hyphal growth.

Figure 5.1.d. Light microscopy (40 x magnification) of *C. albicans* ATCC 10261 incubated on 4 µm x 4 µm x 4 µm grooves. Note hyphal growth is oriented in the same direction as the grooves and ridges.
5.2 Microbial growth on collagen type I coated PMMA discs

When collagen type I was bound to the PMMA substrates and viewed under SEM, it revealed long collagen fibrils connected by shorter fibrils into a flat web-like arrangement. On the grooved substrates, the collagen appeared to have a separate layer on top of the grooved PMMA substrate (Fig 5.2). This may have contributed to no chains being observed along the grooves or the chain growth in the grooves may have been removed by the rinsing of the discs prior to SEM processing to remove any unbound cells.

Figure 5.2. Collagen coated PMMA discs. Note the collagen fibrils are interconnected and appear settled on the ridge.
5.3 *S. gordonii* DL-1

*S. gordonii* DL-1 frequently showed chain growth consisting of more than seven cells on smooth and grooved uncoated PMMA substrates. The chain growth on smooth uncoated discs was randomly orientated (Fig 5.3.a), while that on grooved surface showed chain growth oriented along the grooves (Fig 5.3.b and 5.3.c). The chain growth on grooved discs exhibited aspects of contact guidance along a ridge, over a ridge or along a groove while in contact with the wall and chain growth reoriented to continue growth along the groove-wall interface. These images suggest that *S. gordonii* DL-1, according to the experimental criteria, uses contact guidance to sense and react to the surrounding topography (simulated dentinal tubules).

*S. gordonii* DL-1 chain growth was directed by collagen fibrils on smooth (Figs 5.3.d,e) and grooved (Figs 5.3.f, g, h, i, j, k,l) PMMA discs. On smooth PMMA discs the chain growth was not random because chains grew along collagen type I fibrils. The chains appeared to only have an affinity for the collagen fibrils. Other growth features were noted; collagen appeared to guide chain growth over a wall or ridge when the cells stayed in contact with a collagen fiber (Fig 5.3.f). Chain growth exhibited the characteristics of contact guidance when collagen type I fibrils followed the contours of the grooved substrate. For example, in Figure 5.3.f *S. gordonii* DL-1 incubated on a collagen treated 2µm x 2µm x 2µm grooved surface showed chain growth up a wall and over a ridge while staying in contact with the collagen fibrils. In Figure 5.3.g chained growth, consisting of 3 or more single cells, of *S. gordonii* DL-1 is observed in this SEM image. Out of the 13 chains present, four (31%) appeared to grow along collagen type I at the edges of the groove, six chains (46%) grew along collagen type I up the wall of a groove onto the groove surface and across to the next groove, the remaining three chains (23%) grew along collagen type I but did not meet the criteria for contact guidance. Overall 77% of *S. gordonii* DL-1 cell growth exhibited contact guidance along collagen type I in close proximity to groove edges and over grooves.
However, chain growth did not follow ridges when the collagen fibril was not parallel to or along ridges and grooves (Fig 5.3.j,k,l). The dimensions of the grooves such as groove width and spacing between grooves had no influence on *S. gordonii* DL-1 contact guidance.

Chain growth that was not associated with collagen type I fibrils showed contact guidance growth along the groove wall and ridge (Fig 5.3.m).

The tripeptide appeared to affect the growth of *S. gordonii* DL-1. There was less growth (shorter chains) and fewer cells adhering to collagen type I. The cells present were mostly paired cells or triple cell chains. They appeared to have a high affinity for collagen fibrils rather than to the grooves and ridges. There were a few long chains consisting of several cells adhering to collagen and they appeared to grow along the collagen fibrils (Fig 5.3.n).

There was little evidence of contact guidance growth by the bacteria grown in the presence of the tripeptide.
Figure 5.3.a. *S. gordonii* DL-1 incubated on smooth PMMA discs, note randomly orientated chain growth.

Figure 5.3.b. *S. gordonii* DL-1 incubated on 2 µm x 2 µm x 4 µm grooves, note chain growth along a ridge wall.
Figure 5.3.c. *S. gordonii* DL-1 incubated on 2 µm x 2 µm x 4 µm grooves, note chain growth along the edge of a groove and apparent extension to floor of a groove and re-orientation along the wall.

Figure 5.3.d. *S. gordonii* DL-1 incubated on smooth discs in the presence of collagen type 1.
Figure 5.3.e. *S. gordonii* DL-1 incubated on smooth discs in the presence of collagen type I (image enlarged).

Figure 5.3.f. *S. gordonii* DL-1 incubated on collagen coated 2 µm x 2 µm x 2 µm grooves (image enlarged).
Figure 5.3.g. *S. gordonii* DL-1 incubated on collagen coated 2 µm x 2 µm x 2 µm grooves.

Figure 5.3.h. *S. gordonii* DL-1 incubated on collagen coated 2 µm x 2 µm x 2 µm grooves.
Figure 5.3.i. *S. gordonii* DL-1 incubated on collagen coated 2 µm x 2 µm x 4 µm grooves.

Figure 5.3.j. *S. gordonii* DL-1 incubated on collagen coated 4 µm x 2 µm x 2 µm grooves.
Figure 5.3.k. *S. gordonii* DL-1 incubated on collagen coated 4 µm x 2 µm x 2 µm grooves.

Figure 5.3.l. *S. gordonii* DL-1 incubated on collagen coated 4 µm x 2 µm x 2 µm grooves.
Figure 5.3.m. *S. gordonii* DL-1 incubated on collagen coated 2 μm x 2 μm x 4 μm grooves in the presence of Gly-Pro-Ala.

Figure 5.3.n. *S. gordonii* DL-1 incubated on collagen coated 4 μm x 2 μm x 2 μm grooves in the presence of Gly-Pro-Ala.
5.4 S. mutans NG8

S. mutans NG8 was previously shown to grow in chains on smooth (Fig 5.1.a) and grooved (Fig 5.1.b) PMMA discs. The chains consisted of up to seven cells linked together. Again the SEM images demonstrated that chain growth on smooth PMMA discs was random and direction of growth appeared to be in a disoriented manor. On grooved PMMA discs the cells growing in chains appeared to be growing and reorienting growth along the groove-wall interface and over walls and ridges from one groove to another while staying in contact with the PMMA substrate (Fig 5.1.b). Therefore, in Fig 5.1.b three out of the seven chains reoriented growth along the groove-wall interface, two grew over ridges and two did not meet the criteria of contact guidance. The SEM image suggests that S. mutans NG8 exhibit the characteristics of contact guidance on PMMA discs that simulate the dimensions of dentinal tubules.

Chain growth was directed by collagen type I fibrils on smooth (Fig 5.4.a) and grooved (Fig 5.4.b,c,d) PMMA discs. The chaining growth was similar to that seen in S. gordonii DL-1, being mostly paired cells and long chains consisting of more than 10 cells. The chain growth appeared to exhibit contact guidance when collagen was aligned closely to a groove (Fig 5.4.b), followed the contour of the wall and ridge (Fig 5.4.c) or along the ridge-groove wall interface (Fig 5.4d). Overall the chain growth followed the direction in which the collagen fibrils were aligned on the PMMA substrate.

The dimensions of the grooves such as groove width and spacing between grooves had no influence on S. mutans NG8 contact guidance.

In Gly-Pro-Ala experiments most of the cells present were either single or paired cells (Fig 5.4.e,f), with some short chain growth made up of three cells (Fig 5.2.g). The cells appeared to adhere to collagen, but did not have long chains compared to the mono-culture cells incubated without the Gly-Pro-Ala. These results suggest that the tripeptide Gly-Pro-Ala affected the ability of S. mutans NG8 to adhere to collagen type I fibrils. In addition, the cells that did adhere to collagen did not grow in chains.
Figure 5.4.a. *S. mutans* NG8 incubated on collagen coated smooth PMMA discs. Note the growth is not random in direction but guided by the collagen fibril.

Figure 5.4.b. *S. mutans* NG8 incubated on collagen coated 2 μm x 2 μm x 2 μm grooves. Note the chain growth is along the collagen fibril.
Figure 5.4.c. *S. mutans* NG8 incubated on collagen coated 2 µm x 2 µm x 4 µm grooves

Figure 5.4.d. *S. mutans* NG8 incubated on collagen coated 4 µm x 2 µm x 2 µm grooves
Figure 5.4.e. *S. mutans* NG8 incubated on collagen coated smooth PMMA discs in the presence of Gly-Pro-Ala.

Figure 5.4.f. *S. mutans* NG8 incubated on collagen coated 2 µm x 2 µm x 4 µm grooves in the presence of Gly-Pro-Ala.
Figure 5.4.g. *S. mutans* NG8 incubated on collagen coated 2 µm x 2 µm x 2 µm grooves in the presence of Gly-Pro-Ala.

### 5.5 *E. faecalis* JH2-2

*E. faecalis* JH2-2 was not grown on non-treated PMMA discs as pilot studies showed little bacterial retention.

The cells that exhibited no contact guidance in this study were *E. faecalis* JH2-2. Colonies consisted mostly of single and some paired cells (Figs 5.5.a, b, c, d). No chaining growth was observed using SEM. There was an even and random distribution of cells on smooth and grooved PMMA substrates. It appeared that the presence of collagen type I did not have any effect on *E. faecalis* JH2-2 particularly adherence and guided growth like that shown by *S. mutans* NG8 and *S. gordonii* DL-1.

This suggests that *E. faecalis* JH2-2 did not exhibit contact guidance.

*E. faecalis* JH2-2 was not affected by the tripeptide because the distribution of cells on the smooth and grooved discs not exposed to the tripeptide (Fig
5.5.a,b,c,d) had the same distribution and number of cells to exposed to Gly-Pro-Ala (Fig 5.5.e,f,g)

Figure 5.5.a. Representative image of *E. faecalis* JH2-2 incubated on collagen coated smooth PMMA discs.
Figure 5.5.b. *E. faecalis* JH2-2 incubated on collagen coated 2 µm x 2 µm x 2 µm grooves.

Figure 5.5.c. *E. faecalis* JH2-2 incubated on collagen coated 2 µm x 2 µm x 4 µm grooves.
Figure 5.5.d. *E. faecalis* JH2-2 incubated on collagen coated 4 µm x 2 µm x 2 µm grooves.

Figure 5.5.e. *E. faecalis* JH2-2 incubated on collagen coated 2 µm x 2 µm x 2 µm grooves in the presence of Gly-Pro-Ala.
Figure 5.5.f. *E. faecalis* JH2-2 incubated on collagen coated 2 µm x 2 µm x 4 µm grooves exposed to Gly-Pro-Ala.

Figure 5.5.g. *E. faecalis* JH2-2 incubated on collagen coated 4 µm x 2 µm x 2 µm grooves exposed to Gly-Pro-Ala.
5.6 C. albicans ATCC 10261

C. albicans ATCC 10261 hyphae grew in random directions on smooth PMMA discs (Fig 5.6.a). However the hyphae appeared to avoid other yeast and hyphae, which suggests that the hyphae tip may sense the environment in front of the tip and react accordingly to avoid other cells. In contrast, hyphae growth was guided by collagen fibrils on collagen coated smooth PMMA discs (Fig 5.6.b).

In comparison, almost all the hyphae on grooved PMMA substrate oriented their growth and grew along grooves or ridges (Fig 5.6.c,d). For example, in Figure 5.6.c, C. albicans ATCC 10261 incubated on 4µm x 4µm x 2µm groove dimensions, there were 29 hyphae. Twenty (69%) hyphae reoriented growth along grooves, five (17%) reoriented growth along the edge of a groove and four (14%) did not demonstrate contact guidance. In Figure 5.6.d, C. albicans ATCC 10261 incubated on 4µm x 4µm x 4µm grooves there was 48 hyphae. Ten (20.8%) hyphae grew along grooves, five (10.4%) hyphae grew along the edges, three (6%) hyphae growing along grooves reoriented growth up onto the ridge and the remaining thirty (62.8%) did not fit the criteria for contact guidance along grooved substrates.

Hyphae exhibited the characteristics of contact guidance on ridges where the hyphae growth appeared to be directed in the direction of the ridge (Fig 5.6.e,f). Hyphae were also seen to grow down a groove floor in an acute angle before reorienting tip growth to continue growth along the wall of a groove (Fig 5.6.g,h). Contact guidance appeared to occur more on grooves with shorter spacing between the grooves than grooves with a wider spacing.

Hyphae tended to reorient their tip growth when the angle of approach to a structure was less than 45° (Fig 5.6.e,g,h).

These observations show that C. albicans ATCC 10261 exhibits the characteristics of contact guidance on simulated dentinal tubules in vitro.
C. albicans ATCC 10261 exposed to Gly-Pro-Ala expressed fewer hyphae and were shorter in length. Random orientation was observed on smooth PMMA discs. However, on grooved PMMA substrates the hyphae still exhibited contact guidance. The only difference was that the hyphae were shorter in length. For example, on smooth PMMA discs 8% were observed to have hyphae growth (Fig 5.6.i), that were short in length and direction of growth was random. On a grooved PMMA disc (Fig 5.6.j) there were 42 cells in total on the micrograph image, only 12% extended hyphae and only 5% demonstrated contact guidance along the groove. In Figure 5.6.k. C. albicans ATCC 10261 incubated on 4 µm x 4 µm x 4 µm grooves exposed to tripeptide Gly-Pro-Ala there were 85 cells in total on the micrograph image. A small percentage (3.5%) extended hyphae and only 2% exhibited contact guidance along the ridge of a groove. The results suggest that the tripeptide Gly-Pro-Ala had a considerable effect on C. albicans ATCC 10261 ability to form hyphae.

Figure 5.6.a. C. albicans ATCC 10261 on smooth PMMA discs.
Figure 5.6.b. *C. albicans* ATCC 10261 on smooth PMMA discs in the presence of collagen type I.

Figure 5.6.c. *C. albicans* ATCC 10261 incubated on 4 µm x 4 µm x 2 µm grooves. Note the hyphae reorienting direction of growth was along grooves and ridges.
Figure 5.6.d. *C. albicans* ATCC 10261 incubated on 4 µm x 4 µm x 4 µm grooves. Note hyphae reorienting along grooves and ridges. There are also hyphae not guided by the grooves and ridges.

Figure 4.3.45.6.e. *C. albicans* ATCC 10261 hyphae demonstrating contact guidance along a ridge on 4 µm x 4 µm x 4 µm grooves.
Figure 5.6.f. *C. albicans* ATCC 10261 hyphae demonstrating contact guidance along a ridge on 4 µm x 4 µm x 4 µm grooves.

Figure 5.6.g. *C. albicans* ATCC 10261 hyphae demonstrating contact guidance along a groove wall on 4 µm x 4 µm x 2 µm grooves.
Figure 5.6.h. *C. albicans* ATCC 10261 hyphae demonstrating contact guidance along a groove wall on 4 µm x 4 µm x 2 µm grooves.

Figure 5.6.i. *C. albicans* ATCC 10261 incubated on smooth PMMA discs exposed to tripeptide Gly-Pro-Ala.
Figure 5.6.j. *C. albicans* ATCC 10261 incubated on 4 µm x 4 µm x 2 µm grooves exposed to tripeptide Gly-Pro-Ala.

Figure 5.6.k. *C. albicans* ATCC 10261 incubated on 4 µm x 4 µm x 4 µm grooves exposed to tripeptide Gly-Pro-Ala.
6 DISCUSSION

Grooves were created on plastic PMMA discs to simulate some characteristics of dentinal tubules. Collagen type I was also used to coat the discs to better replicate a simulated dentinal tubule environment.

Miller (1890) was the first to demonstrate bacterial invasion of dentinal tubules of both carious and non-carious dentine and he reported that the tubule microflora consisted of cocci and rods. In the current research PMMA discs were used. PMMA was used because the structure can be manufactured into a standardized form, whereby the grooves and spacing between the grooves remain constant, allowing a close resemblance to the longitudinal characteristics of dentinal tubules. No published studies have used PMMA as a substrate to study oral microbe infection and invasion in vitro. However, several in vitro studies have used dentine from extracted human teeth (Michelich et al. 1980; Akpata & Blechman 1982; Meryon et al. 1986; Meryon & Brook 1990; Nagaoka et al. 1995a; Love 1996a; Love 1996b; Love et al. 1997; Berkiten et al. 2000; Love et al. 2000), bovine teeth (Haapasalo & Ørstavik 1987; Perez et al. 1993; Siqueira et al. 1996) and ferret teeth (Meryon et al. 1986).

6.1 Streptococcus mutans NG8

*S. mutans* is well known as the predominant bacteria in the oral cavity (Nyvad & Kilian 1990) and is implicated in dentinal tubule invasion in vitro (Siqueira et al. 1996; Love et al. 1997).

In the initial pilot study, *S. mutans* NG8 did not grow on the PMMA surface when placed on top of agar regardless of incubation time which confirms bacteria need to be in direct contact with a source of nutrition (in this case TSBY agar) for growth and colonization. In comparison, when *S. mutans* NG8 was inoculated into a broth of BHY, the SEM images revealed growth on the blank and grooved discs. The cells were inoculated in broth before being introduced into the wells containing the PMMA discs. This ensured that cells were
randomly settling on the PMMA discs before incubation (Kouchi et al. 1980). The cells were typically observed in chains consisting of several single uniformed shaped cells, paired or single cells on both PMMA smooth and grooved discs. However, the majority of cells present were either single, paired or in colonies which may have affected or influenced the cells to initiate chaining growth. A likely reason for this may be the interaction between cells with the PMMA substrate. Hisada (1976) demonstrated in an in vitro study the adhesion of mouse fibrblasts to various thermoplastic plastic coated coverslips, such as polyvinyl acetate, cellulose, polymethylmethacrylate, polystyrene and polyvinyl alcohol. Under light microscopy the best substrate for cell adhesion was polyvinyl acetate followed by cellulose, polymethylmethacrylate and polystyrene. This earlier study suggests that polymers may influence cell adhesion and subsequently may also affect the way cells grow on these surfaces.

Qualitative analysis showed S. mutans NG8 growth in random directions on smooth (Fig 5.1.a) PMMA discs. Some chains exhibited characteristics of contact guidance (Fig 5.1.b) along the walls of grooves and over ridges. With the grooved substrate, chaining growth was evident with cells growing along the floor of a groove against the wall, cells growing along a groove encountering a wall, then re-orientating and continuing growing along the wall, and cells growing over a wall and ridge whilst staying in close contact with the substrate. In addition, the width and spacing of the grooves did not influence cell growth. This supports the concept that contact guidance is a response to the change in the topography such as that guided by grooves, ridges, scratches and pores (Sherwood et al. 1992; Gow et al. 1994a; Curtis & Wilkinson 1997).

The question now is whether collagen is a preferred substrate for adhesion and is the chain growth along fibrils contact guidance or is it following the proposed model by Love and associates (1997) of adhesion-upregulation and chain growth when associated with collagen.

The SEM images clearly reveal that S. mutans NG8 growth was random and did not demonstrate contact guidance on smooth PMMA discs. However, S. mutans NG8 grew in chains along collagen type I fibrils on both smooth (Fig 5.4.a) and
grooved (Fig 5.6.b.c,d) PMMA discs. The *S. mutans* NG8 chain growth appeared to be guided by collagen type I fibrils when samples were viewed under SEM. This is similar to that described by Love *et al.* (1997) who demonstrated that cells exhibited binding to collagen and a growth response to collagen mediated by antigen I/II proteins, and confirmed by Heddle *et al.* (2003). Chaining growth over walls and ridges (Fig 5.4.c) and along grooves (Fig 5.4.d) only occurred when the collagen fibrils were parallel or perpendicular to the grooves. This suggests that collagen not only provides a substrate to which bacteria can adhere to but as a guide for bacteria to grow along resulting in the invasion of dentinal tubules and root canals (Akpata & Blechman 1982; Haapasalo & Ørstavik 1987; Ørstavik & Haapasalo 1990; Perez *et al.* 1993; Nagaoka *et al.* 1995b; Love *et al.* 1996; Love *et al.* 1997).

Dai *et al.* (1991) showed that unmineralized collagen was a major component within dentinal tubules and it is unrelated to age, suggesting that collagen is continually laid down within tubules throughout life. Therefore, it is available as an adhesion substrate throughout life and this supports the notion of collagen acting as a bacterial adhesin and growth modifier.

However, the collagen fibrils in this *in vitro* study were arranged in a woven mesh-like structure or as long straight strands, which is different to the arrangement of collagen fibrils in dentinal tubules. Dentinal tubules are lined with collagen fibrils associated with the intratubular dentinal walls. SEM images showed that etched dentine exposes strands of collagen with spaces (Bolla *et al.* 2000). The spacing may also affect bacteria chaining growth down dentinal tubules.

*S. mutans* NG8 exposed to the tripeptide Gly-Pro-Ala did not demonstrate the characteristics of contact guidance when compared to the cells not exposed to the tripeptide. The cells did not exhibit any chaining growth along collagen strands, grooves and ridges. Most of the cells were now in pairs instead of chains. This tripeptide had a negative effect on *S. mutans* NG8 growth and its ability to grow in chains. In this particular *in vitro* study, Gly-Pro-Ala appeared to affect *S. mutans* NG8 chain growth and adhesion of cells onto collagen type I
fibrils and this may represent competitive binding of the tripeptide. There has been no published data on this and further quantitative studies are needed to confirm these findings.

6.2 Streptococcus gordonii DL-1

*S. gordonii* DL-1 chain growth was random on smooth (Fig 5.3.a) uncoated PMMA discs. In comparison, the SEM images showed *S. gordonii* DL-1 chain growth was guided along non-coated grooved PMMA discs (Fig 5.3.b and 5.3.c). The chain growth exhibited contact guidance staying in contact along the ridge and groove floor. This suggests that the bacteria cell wall is interacting with the PMMA substrate. There was no other variable that may have influenced this type of growth behaviour. The cells were continually surrounded by a rich supply of nutrients and they were incubated in a static position without shaking. The only possible cause that influenced the chain growth is the change in the topography (Sherwood et al. 1992; Gow et al. 1994a; Curtis & Wilkinson 1997).

The chaining growth in this study followed the shape of collagen strands (Fig 5.3.e). However, when collagen stayed in contact and follows the substrate, the chain growth was guided along the collagen taking the form of the substrate (Fig 5.3.f). The majority of collagen settled on top of the grooved substrate and did not appear to settle down inside the grooves, which may have accounted for the absence of cells growing along the actual grooves. The absence of cells along the grooves devoid of collagen fibrils confirms the findings by Love et al. (1997) and Heddle et al. (2003) that streptococci bind to collagen through cell surface proteins.

The addition of tripeptide (Gly-Pro-Ala) considerably affected adhesion and overall length of *S. gordonii* DL-1 chains. Although the chains were shorter in length, the chains still exhibited contact guidance on collagen that was aligned along the grooves. Heddle et al. (2003) suggested a significant increase in *S. gordonii* DL-1 adhesion to SAG (gp340) on plastic surfaces when exposed to the tripeptide Gly-Pro-Ala. However, the result for this part of their research was not
shown. These findings may prove Gly-Pro-Ala to be ineffective against *S. gordonii* DL-1 *in vivo* because collagen may be coated in SAG that has been shown to increase binding in the presence of Gly-Pro-Ala tripeptide. However, SAG is a salivary protein and therefore it is not likely to be present in radicular dentinal tubules. Furthermore, Gly-Pro-Ala could also be seen as a competitive antagonist to collagen which may be of interest when developing novel agents against bacteria invasion into dentinal tubules.

### 6.3 Enterococcus faecalis JH2-2

*E. faecalis* is the most tenacious bacteria to endodontists because it has been associated with persistent lesions and failed root canal treatments despite following strict protocols (Sundqvist 1992b; Molander *et al.* 1998; Sundqvist & Figdor 2003; Rocas *et al.* 2004; Sedgley *et al.* 2006).

In this research, *E. faecalis* JH2-2 was used because this strain has been shown *in vitro* to form moderate to heavy tubule invasion (Love 2001; Chivatxaranukul *et al.* 2008). Of the three bacteria used in this research, *E. faecalis* JH2-2 was the only one that did not exhibit any of the criteria of contact guidance on plain or grooved PMMA discs or grooved discs coated with collagen type I. The cells were mostly single or paired with random and sparse distribution over the substrate. *E. faecalis* JH2-2 did not appear to have any affinity to the surrounding collagen (Fig 5.5.a,b,c,d).

Chivatxaranukul *et al.* (2008) had similar SEM images of *E. faecalis* JH2-2 incubated on extracted intact human teeth that were split in half and incubated over a period of eight weeks. Instead of using BHI similar to what was used in this *in vitro* study, they used chemically defined medium that did not contain proteins or peptides but consisted of amino acids, vitamins and minerals plus glucose. The bacterial adhesion to the tubule walls was significantly and consistently less than the adhesion to fractured intertubular dentine. Even when collagen fibres lining the intratubular walls were clearly visible, little adherence occurred. Other factors may have influenced *E. faecalis* JH2-2 colonization on
the PMMA discs such as nutrient conditions, collagen structure and ability of bacteria to proliferate as chains (Love et al. 1997).

In addition, *E. faecalis* JH2-2 colonisation was not affected by the Gly-Pro-Ala tripeptide. The distribution of the cells on the PMMA discs was the same as those without the tripeptide. This suggests that this tripeptide may only affect early colonizing bacteria as seen earlier in *S. mutans NG8* and *S. gordonii* DL-1.

### 6.4 Candida albicans ATCC 10261

*C. albicans* is identified as the most dominant oral yeast species (Egan et al. 2002; Waltimo et al. 2003). In this study, *C. albicans* ATCC 10261 exhibited the characteristics of contact guidance along collagen (Fig 5.6.b), along ridges (Fig 5.6.e and 5.6.f) and groove walls (Fig 5.6.g and 5.6.h). Hyphae were observed to grow along collagen fibrils on coated smooth PMMA discs. In comparison, on uncoated smooth PMMA discs, the hyphae grew in random directions with no specific direction of growth. This suggests that collagen guides the growth of hyphae along a smooth surface. This may be difficult in dentinal tubules because the orientation of collagen fibrils are short and mostly perpendicular to the direction of the dentinal tubule (after etching to remove the smear layer) with spaces between each collagen fibril (Bolla et al. 2000). An earlier paper by Dai et al. (1991) suggested otherwise using SEM, where they demonstrated collagen fibrils lying parallel to the tubule consisting of single fibrils, loosely bundled fibrils to densely packed fibrils.

The arrangement of collagen fibrils may contribute to the low number of *C. albicans* strains reported to have been isolated from root canals (between 3% to 40%). *C. albicans* had different patterns of radicular dentin infection. In some specimens, colonization was slight and no dentinal tubule penetration was observed. However, in other specimens, some areas of the root canal walls were covered with large colonies and some dentinal tubules were heavily infected (Siqueira et al. 2002). This suggests that the presence of collagen may affect the ability of Candida to invade dentinal tubules.
Sen et al. (1997a) demonstrated that when smear layer was present on dentine, there was a thick biofilm of \textit{C. albicans}. In addition, collagen may be covered by smear layer which may interfere with \textit{C. albicans} tubule invasion. In contrast, when the smear layer was absent, a biofilm was not present although separate and distinct colonies were present. In another study by the same authors, they demonstrated that the presence of smear layer increased the adhesion of \textit{C. albicans} to dentine (Sen et al. 2003).

\textit{C. albicans} ATCC 10261 was grown on substrates with two different groove dimensions 4 µm x 4 µm x 2 µm (WDS) and 4 µm x 4 µm x 4 µm (WDS) to determine whether the change in the substrate topography influences the growth of hyphae. Analysis suggests that there was a difference between the two substrates (Fig 5.6.c and 5.6.d). On a 4 µm x 4 µm x 2 µm (WDS) grooved surface, 69% of the hyphae reoriented growth along a groove compared to those on 4 µm x 4 µm x 4 µm grooves where 20.8% hyphae reoriented growth along grooves, 10.4% hyphae reoriented growth along edges, 6% hyphae growing along grooves reoriented growth up onto the ridge and the remaining 62.8% did not fit the criteria for contact guidance along grooved substrates. This is in agreement with earlier papers that contact guidance is guided by the topography of the surface (Sherwood et al. 1992; Gow et al. 1994a; Curtis & Wilkinson 1997).

Watts et al. (1998) demonstrated hyphae grown on polystyrene membranes with ridges of 1.53 µm height and 20 µm pitch, and showed greater than 82-90% of hyphae reoriented the direction of growth on contact with a ridge when the approach angle was 0-30°. They also noticed a trend that as the angle of approach increased, the number of hyphae reorienting their tip growth decreased. At an angle of 71-90°, there was no reorientation in growth direction. In this study, the hyphae tip reoriented growth when the angle of approach was less than 45° and more hyphae reoriented tip growth when the approach was less than 30°. These findings are similar to Watts and colleagues (1998). However, yeast on a groove edge produced hyphae that did not reorient growth (Fig 5.6.f). The tip emerged straight from the yeast as if the cell sensed the edge, continuing.
growth along the groove edge. This confirms that *C. albicans* ATCC 10261 exhibits the characteristics of contact guidance on simulated dentinal tubules *in vitro*.

A possible cause for the change in hyphae tip growth has been suggested to be from stretch-activated channels (Watts *et al.* 1998; Brand *et al.* 2007). In this research there was no source of calcium ions to facilitate hyphae reorientation. Therefore, this means that there are other mechanisms which drive *C. albicans* ATCC 10261 contact guidance characteristics.

Lastly, the tripeptide Gly-Pro-Ala affected hyphal growth and propagation on smooth and grooved surfaces. There were significantly fewer hyphae that were shorter than the unexposed group. However, this did not affect the contact guidance of hyphae. This suggests that the tripeptide Gly-Pro-Ala affected the production of hyphae by *C. albicans* ATCC 10261 in this *in vitro* study.

The first hypothesis (1) was proven and the second (2) was not proven, because some bacteria did exhibit characteristics of contact guidance when growing as chains:

1. *C. albicans* ATCC 10261 will exhibit contact guidance (thigmotropism) along simulated dentinal tubules.

2. Since bacteria are proteolytic, they will not exhibit contact guidance.
6.5 Limitations of the research

There were shortcomings in this research regarding contact guidance using three selected bacteria and *C. albicans* as the control. The major limitation was the possible removal of collagen and cells from the PMMA substrate during rinsing with PBS and GSB to remove any unbound cells from the substrate. This may have affected the interpretation of SEM images. In the initial pilot study, the PMMA discs were not rinsed with PBS to prevent any loss of cells. The pilot study, as stated earlier, was performed to determine whether the selected organisms can grow on PMMA discs on an agar medium or in a suspension of growth medium. The broth showed positive results, so PMMA discs were rinsed in the main experiments to remove any unbound cells.

Another drawback was the use of two dimensional images captured with SEM. Different angles of the images may have demonstrated the contact between collagen and bacteria/yeast more accurately along the entire length of chains or hyphae. The SEM also did not have a higher resolution so sharp details could not be magnified past a certain limit; in this study a magnification beyond 2500x became increasingly unclear and poor quality.

6.6 Impact on future research

This study has provided a foundation on which future quantitative studies can improve and refine research into contact guidance by oral microbes and identify other possible molecules that can inhibit dentinal tubule invasion. Because it is not possible to eliminate all microbes from the oral cavity, it is essential to develop novel means of preventing invasion of dentinal tubules.
This study was carried out to determine whether *E. faecalis* JH2-2, *S. mutans* NG8 or *S. gordonii* DL-1 exhibit the characteristics of contact guidance along polymethylmethacrylate (PMMA) discs.

Within the scope of this research, it was identified that *S. gordonii* DL-1 and *S. mutans* NG8 do exhibit some of the characteristics of contact guidance on PMMA discs *in vitro* when growing as chains. *E. faecalis* JH2-2 did not exhibit contact guidance on smooth or grooved PMMA discs. The control organism, *C. albicans* ATCC 10261 confirmed contact guidance on simulated dentinal tubules (PMMA discs).

SEM provides an effective method of capturing the growth patterns of oral microbes on PMMA discs. The PMMA discs closely resembled dentinal tubules in terms of dimensions.

The Glycine-Proline-Alanine tripeptide had an effect on cell adhesion and length of chaining growth in *S. gordonii* DL-1 and *S. mutans* NG8, and hyphae formation in *C. albicans* ATCC 10261. The tripeptide had no effect on *E. faecalis* JH2-2.
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