Development of an in vivo model to investigate the effects of three adhesive removal methods around orthodontic brackets: A pilot study

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Abstract

Background

Orthodontic treatment has increased significantly over the last two decades, however the prevalence of biofilm related complications in the form of white spot lesions (WSLs) is still unquestionably prevalent. Orthodontic adhesives have been shown to be a critical factor for bacterial attachment and proliferation. With the increasing use of indirect bonding systems, advances in adhesive technology and lack of well-designed clinical trials, there is uncertainty regarding clinical guidelines for the management of excess adhesive around orthodontic brackets.

This study’s primary objectives were to develop a protocol to compare the effects of three removal methods of excess adhesive around enamel-bonded orthodontic brackets on the acidogenicity of the formed biofilm following 96 hours in vivo.

Methods

A prospective study involving participants wearing customised intraoral appliances containing six randomised bovine enamel discs (three on each side) with bonded orthodontic brackets to facilitate intraoral biofilm development.

Validation of pH acidogenicity was initially performed with a *Streptococcus mutans* UA159 biofilm formed in vitro on bovine enamel discs containing orthodontic brackets and compared to stainless steel brackets on their own, following a glucose (10% w/v) challenge. The ion dissociation of sterile enamel discs was also tested on the pH assay.

To test the validity of a maxillary double vacuum-formed appliance, a volunteer wore the appliance continuously for four days containing enamel-bonded orthodontic brackets. Ninety-six hours was established as the optimum time for mature biofilm formation.
Eight healthy adult volunteers (age range = 25-39) wore customised maxillary double vacuum-formed appliances containing six bovine enamel discs (three on each side) with bonded orthodontic brackets for 96 hours. The three adhesive removal interventions (bur, no-removal and scaler) were randomised into canine, premolar or molar positions. The appliances were immersed in a sucrose solution (10% w/v) five times a day to facilitate biofilm growth.

pH measurements were done ex vivo on all biofilm bearing discs and control discs (absence of bacteria) following a glucose challenge (10% w/v). Cross sectional microhardness and elastic modulus measurements were obtained at the composite enamel interface and at the internal control (underneath the bracket).

**Results**

Six volunteers completed the study. There was no difference between adhesive removal methods on final pH after glucose challenge. There was a significantly lower final pH for the discs located at the premolar position (5.3 ± 0.1; p=0.006) and right side of the mouth (5.3 ± 0.1; p=0.009) compared to control discs (7.3 ± 0.2; p<0.001).

There was no significant difference between the interventions for the microhardness and elastic modulus at the enamel-composite interface. There was a significant difference for the microhardness and elastic modulus at the internal control (underneath the bracket) between the scaler, bur and no removal interventions (p≤0.05) and canine, premolar and molar positions (p<0.05).

**Conclusion**

There is no significant difference between adhesive removal method around orthodontic brackets on the formation of an acidogenic biofilm in low risk individuals. The location in the mouth is highly correlated with creation of a cariogenic biofilm.
Acknowledgements

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This research would not have been conceivable without the help from the PC2 Lab staff and Biomaterials lab. A special thank you to Dr Kc Li and Dr Carolina Loch for your technical guidance and sharing your knowledge about Nanoindentation.

Thank you to all the study participants that took part in this research, without you this project would not have been possible. To my colleagues, thank you for all the banter, support, and endless laughs that have made this testing experience an enjoyable one. To my classmates, Divya and Caleb, I could not have chosen two better people to share this experience with. Thank you both.

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encouraging when I doubted myself, giving me a push when I needed it and being there when I couldn’t be. You are my rock, thank you for everything. To my kids Olivia and Tommy. I love you both beyond words, your energy, kindness and zest for life makes me so proud to be your mama.
Preface

This three year Doctoral Thesis was completed as partial fulfillment of the Degree of Clinical Dentistry. The thesis describes the research conducted in the Department of Oral Sciences, Discipline of Orthodontics, University of Otago, New Zealand. It contains work conducted between January 2016 and August 2018.

An abstract for this study was accepted for presentation at the Sir John Walsh Institute Research Day, August 2018.


The candidate was responsible for:

- Development of the study concept and design in conjunction with her supervisors.
- Consulting with the University of Otago Human Ethics Committee regarding ethics protocol.
- Completing the ethics and Maori consultation process.
- Applications for funding.
- Recruitment of participants for the study.
- Testing of the intraoral appliance prototypes.
- Fitting of the intraoral appliance and enamel discs.
- Extraction of bovine teeth, cutting and polishing enamel discs.
- Calibration of the pH meter.
- Developing and undertaking the protocol for assessment of plaque acidogenicity.
- Embedding of discs, cutting and polishing for microhardness testing.
- Analysis of discs with nanoindentation.
• Analysis of nanoindentation results with software.
• Statistical analysis in conjunction with Professor Farella.
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<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
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<tr>
<td>cGy</td>
<td>Centigray</td>
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<tr>
<td>C. albicans</td>
<td>Candida albicans</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>Ca₁₀(PO₄)₆(OH)₂</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
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<td>E</td>
<td>Elastic modulus</td>
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<td>GPa</td>
<td>Gigapascal</td>
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<td>H</td>
<td>Hardness</td>
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<tr>
<td>H₂</td>
<td>Hydrogen molecule</td>
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<td>H⁺</td>
<td>Hydrogen ion</td>
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<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>HPO₄²⁻</td>
<td>Hydrogen phosphate ion</td>
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<tr>
<td>ICT</td>
<td>Intra-oral cariogenicity</td>
</tr>
<tr>
<td>kGy</td>
<td>Kilogram</td>
</tr>
<tr>
<td>OH⁻</td>
<td>Hydroxyl ion</td>
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<tr>
<td>N</td>
<td>Force</td>
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<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NaHOCL</td>
<td>Sodium hypochlorite</td>
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<tr>
<td>P-h</td>
<td>Load - displacement</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>Phosphate ion</td>
</tr>
<tr>
<td>RMGIC</td>
<td>Resin-modified glass ionomer cement</td>
</tr>
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<td>RMO</td>
<td>Rocky mountain orthodontics</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<td>S. mitis</td>
<td><em>Streptococcus mitis</em></td>
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<td>S. mutans</td>
<td><em>Streptococcus mutans</em></td>
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<td>S. oralis</td>
<td><em>Streptococcus oralis</em></td>
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<tr>
<td>S. sangius</td>
<td><em>Streptococcus sanguis</em></td>
</tr>
<tr>
<td>S. salivarius</td>
<td><em>Streptococcus salivarius</em></td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<td>SFE</td>
<td>Surface free energy</td>
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<tr>
<td>UMIS</td>
<td>Ultra-Micro-Indentation-System</td>
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<tr>
<td>w/v %</td>
<td>weight/volume %</td>
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<td>WSL</td>
<td>White spot lesion</td>
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Chapter 1

Literature Review
1.1 Introduction

Orthodontic treatment has increased significantly over the last two decades with patients seeking to improve facial aesthetics, oral function and social wellbeing. A recent review from the American Association of Orthodontists reported that the number of patients seeking orthodontic treatment has doubled in the last 30 years (American Association of Orthodontists, 2012). Although advances in orthodontic materials have improved clinical efficiency and patient comfort, enamel demineralisation in the form of white spot lesions (WSLs) at the bracket-adhesive enamel interface is still unquestionably prevalent (Ren et al., 2014). This undesired effect is concurrent with orthodontic treatment resulting from extended plaque accumulation on the affected surfaces due to poor oral hygiene (Hu & Featherstone, 2005).

WSLs are defined as a subsurface enamel porosity from carious demineralisation which presents as a milky white opacity when located on smooth surfaces (Summitt et al., 2001). Enamel translucency is directly related to the degree of mineralisation. The increase in subsurface enamel porosity as a result of demineralisation changes the optical properties of the enamel resulting in a loss of translucency which makes the enamel appear more opaque. Additionally, the uneven surface changes resulting from direct erosion of the outermost layer of enamel results in a diminished reflection of light (Fejerskov et al., 2015). The reported incidence of WSLs is estimated to be present in 50%-97% of orthodontic patients compared to 11%-24% of matched controls (Boersma et al., 2005; Gorelick et al., 1982; Lapenaite et al., 2016). Although some WSLs are capable of remineralising to a visually sufficient appearance, if left to persist they can lead to permanent unaesthetic enamel scars (Ren et al., 2014). Early termination of treatment may also be required resulting in compromised clinical outcomes. In severe cases, extensive restorative dental treatment is needed, further increasing the financial burden on patients (Ren et al., 2014).
Fixed orthodontic appliances create stagnation sites for bacterial accumulation and make oral hygiene challenging (Mei et al., 2011). The adhesion of bacteria is most prevalent on orthodontic adhesives compared to other orthodontic materials (Gwinnett & Ceen, 1979; Lim et al., 2008; Sukontapatipark et al., 2001). The initial bacterial forces are stronger to adhesive compared to orthodontic brackets and enamel (Mei et al., 2009). Excess orthodontic adhesive that has not been effectively removed around the orthodontic bracket is particularly prone to rapid biofilm accumulation and maturation (Sukontapatipark et al., 2001). The most critical factor in plaque aggregation is the surface area of composite resin that is exposed surrounding an orthodontic bracket (Gwinnett & Ceen, 1979). In addition, the wear of the resin matrix in orthodontic adhesives exposes the filler particles increasing the surface area and roughness further enhancing the rapid attachment and proliferation of oral bacteria (Gwinnett & Ceen, 1979). Microscopic gaps resulting from polymerisation shrinkage of the adhesive further contribute to plaque accumulation at the adhesive-enamel interface, providing protection for bacteria against oral cleansing forces (Ogaard, 2008; Sukontapatipark et al., 2001).

The dental plaque not only increases in volume but also in its cariogenic properties following placement of orthodontic fixed appliances. There is an increase in the levels of mutans streptococci and periodontopathic bacteria including Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens and Fusobacterium species (Al Mulla et al., 2009; Y. Liu et al., 2013). Placement of orthodontic fixed appliances also results in the reduction of the pH of plaque and saliva compared to non-orthodontic patients (Arab et al., 2016; Balenseifn & Madonia, 1970; Chang et al., 1999).
1.2 Dental Enamel

Human dental enamel is a highly mineralised acellular tissue which is composed of 99% of calcium phosphate crystals by weight (Fejerskov & Larsen, 2015). Due to its highly mineralised content, the physical, especially hardness, properties of enamel are comparable to mineral hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. The calcium ($\text{Ca}^{2+}$), phosphate ($\text{PO}_4^{3-}$) and hydroxyl ($\text{OH}^-$) ions of enamel crystals are arranged in a repeating crystal lattice framework very similar to hydroxyapatite (HA). The enamel crystals are further divided into rod and interrod regions based on their orientation, with the long axes of the crystals in the rod region running parallel to the rod direction and the interrod crystals gradually changing their orientation from parallel to perpendicular in the deepest parts of the interrod region. This pattern is often referred to as the “fish shape” comprising of the body (B) rod region and tail (T) interrod region (Figure 1 (Meckel et al., 1965)).

![Figure 1. Electron micrograph of cross-section of enamel rods in mature human enamel. Crystal orientation is different in “bodies” (B) than in “tails” (T). Approximate level of magnification 5000× (Meckel et al., 1965)](image)
The interrod spaces are composed mainly of water and organic material. These form a network of passages for the diffusion of molecules (Kidd, 2004). These intercrystalline spaces are important in the initial stages of WSL formation through the opening up of the spaces following acid exposure and route for subsequent mineral loss during the demineralisation process (Holmen et al., 1985).

The surface of enamel is irregular having grooves also known as “perikymata”, Tomes’ process pits, development focal holes and fissures. Importantly, the surface of enamel undergoes a significant amount of biochemical change in its post-eruptive maturation stage (Fejerskov, 2015). During eruption, there is an increased load of bacterial accumulation on the tooth surface due to difficulty in mechanical removal of plaque in this area and retentive surfaces provided by the erupting tissues. The resulting biofilm causes dynamic pH fluctuations at the surface of enamel which can be seen as areas of dissolution of rod and interrod regions on microscopic examination resulting in a “subclinical” caries lesion. With the continued passive eruption of the tooth, reorganisation of gingival fibres and establishment of the interproximal and occlusal contacts, the local environment is greatly improved and the lesion arrests (Fejerskov, 2015). This has previously been described as a period of “passive mineral uptake”, however, it may be more correctly referred to as post eruptive secondary maturation (Fejerskov et al., 2015).

1.2.1 Enamel Dissolution

Dental enamel can have varying percentages of carbonate and fluoride integrated into the lattice structure. The proportions of these two components vary between individuals and amongst teeth and they have differing effects on enamel solubility. The increasing carbonate results in greater dissolution of HA while the opposite effect occurs with increasing incorporation of fluoride (Moreno et al., 1974; Nelson, 1981).
When enamel is in contact with pure water, the water molecules cause dissociation of ions from the crystal lattice framework. For every unit of solid HA, there is a resultant five Ca$^{2+}$, three PO$_4^{3-}$ and one OH$^{-}$ that are discharged. This process continues either until the water is saturated with HA or until the pH rises (Fejerskov & Larsen, 2015).

Acid (H+) influences the rate of dissolution. The H+ react with the PO$_4^{3-}$ and OH$^{-}$ to form hydrogen phosphate (HPO$_4^{2-}$) and water (H$_2$O). This results in the solution becoming unsaturated, causing further dissolution of HA until the solution re-equilibrates.

The net loss of mineral increases by a factor of 10 with each unit of pH decrease. Once a certain level of mineral loss is achieved, the resultant increase in porosity is evident clinically as a WSL (Fejerskov & Larsen, 2015).
1.3 Oral Biofilm

Most microorganisms grow naturally as biofilms attached to surfaces. Oral biofilms can be defined as matrix-embedded microbial populations, adherent to each other and/or to surfaces or interfaces (Costerton et al., 1995). Biofilms are the preferential mode of growth for most bacterial species, as biofilms provide several advantages compared to single-cell (planktonic/sessile) bacteria including defense against environmental factors, host factors and competing microorganisms. Furthermore, bacterial species present in biofilms have a complex synergistic relationship and their ability to alter their local environment by modifying gene expression, promoting nutrient uptake and removal of toxic substances through metabolism by other bacteria make them very challenging therapeutic targets. The formation of biofilms on the hard and soft tissues in the oral cavity results in the most common infections in humans, dental caries and periodontal disease (Marsh, 2005; Socransky & Haffajee, 2002). On tooth surfaces, the mildest form of this disease is WSLs. With increasing severity this can result in large areas of enamel loss and breakdown. On the soft tissues, particularly if persistent in gingival pockets, biofilms can lead to periodontal disease and tooth loss (Ren et al., 2014). The feature distinguishing caries and periodontal disease from other human infectious diseases is that the causative agent is generally an endogenous biofilm (Socransky & Haffajee, 2002).

1.3.1 Oral Biofilm Structure

The composition of oral biofilms is approximately 15-20% bacterial cells by volume arranged in a complex heterogenous distribution of microcolonies embedded in a matrix or glycolyx (Socransky & Haffajee, 2002). The matrix is composed of exopolysaccharides, proteins, water and solutes. The exopolysaccharides are secreted by the bacteria and make up the bulk of the dry weight of the biofilm (50-95%). This extracellular matrix acts as the “backbone of the biofilm” (Socransky & Haffajee, 2002). It offers protection against
antimicrobial substances and environmental factors, acts as a buffer for bacterial enzymes and gives the biofilm its structure (Flemming & Wingender, 2010). It also constitutes an effective adhesive. A differentiating characteristic of oral biofilms is that the majority of bacteria not only synthesise the exopolysaccharides but also degrade them (Socransky & Haffajee, 2002).

Earlier electron microscopy studies reveal that supragingival dental plaque is a homogenous structure composed of a combination of microbial species (Listgarten, 1976; Theilade et al., 1976). However, the preparation methods required for electron microscopy alter the natural structure of the biofilm through fixation and drying of the specimens. Confocal microscopy has overcome this limitation as the oral biofilm is studied in its natural hydrated environment. These studies demonstrate that biofilm architecture is profoundly heterogenous, composed of cells, matrix and channels in a distinct spatially organised structure (Wood et al., 2000). The channels or pores of biofilms have been described as a primitive circulatory system, as they provide a connection from the oral environment to the tooth surface and allow the movement of nutrients and other ions throughout the biofilm (Auschill et al., 2001; Wood et al., 2000). There is a reduction in the number of these channels as the biofilm matures, which reduces the diffusion of ions into and out of the biofilm resulting in persistent acid presence adjacent to the enamel surface (Characklis et al., 1990). Bacterial viability staining demonstrates that more viable bacteria are present near the channels (Auschill et al., 2001). However, the nature of interaction between the various exo-polymers secreted by the bacteria in the matrix and their effect on the diffusion of ions in and out of the channels is still poorly understood (Robinson et al., 1997).

Environmental factors including nutrients, antimicrobial substances, oxidation-reduction potential and pH are particularly important to the growth of bacteria, however they do not display a linear concentration gradient in the biofilm (Marsh, 2005). A study using micro-electrodes and two-photon excitation
microscopy demonstrated that pH varies remarkably within a biofilm. This in vitro study used a mixed model culture consisting of ten bacterial species and revealed that after a sucrose challenge, bacterial colonies with pH below 3.0 were neighbouring bacterial colonies with a pH above 5.0 (Vroom et al., 1999). This may help to explain how bacteria with different metabolic needs can survive in a biofilm.

1.3.2 Oral Biofilm Formation

Oral biofilm forms as a sequence of ordered events and stages. The formation of the pellicle marks the preliminary stage of biofilm development. The pellicle is composed of salivary glycoproteins, lipids and constituents of the gingival crevicular fluid (Marsh et al., 2015). The pellicle has several contrasting functions; it acts as a defense barrier through its lubrication, anti-erosive, buffering properties and various antibacterial proteins i.e. lactoferrin, cystatins and lysozyme (Deimling et al., 2007; Hannig et al., 2005; Hannig et al., 2004; Pruitt et al., 1969). However, the pellicle also provides receptors for bacterial adhesion (Hannig & Hannig, 2009). The initial colonising bacterial attachment to the pellicle is mediated by bacterial fimbriae as well as short interaction forces (electrostatic interactions, ionic interactions and Lewis acid-base interactions), medium range (hydrophobic interactions) and long range interactions (Van der Waals and Coulomb forces) which pass through a phase of reversible interactions before establishing irreversible bonds and attachment (Hannig & Hannig, 2009).

The initial bacteria are mostly streptococci (S. sanguis, S. oralis, S. mitis) which form an attachment to the pellicle within just four hours. Actinomyces and gram-negative bacteria are often also present. Although the mutans streptococci form only about 2% of the initial colonisers they thrive in acidic conditions and produce extracellular polysaccharides which block the diffusion of acid from the enamel surface promoting enamel demineralisation (Nyvad &
Kilian, 1987). The next stage in development is growth and formation of microcolonies over twenty-four hours. With time, the plaque becomes more dominated by Actinomyces species through microbial succession and the microbiota becomes more diverse (Syed & Loesche, 1978). A study utilising glass pH sensors in a sample of three patients about to undergo fixed appliance treatment showed that two day old plaque has the ability to affect the pH gradient following a sucrose challenge below the critical pH of 5.5 and cause demineralisation of the enamel (Imfeld & Lutz, 1980). This finding should be interpreted with caution as the sample size was extremely small (three patients) and the appliance which housed the electrode was acrylic which tends to grow a plaque different compared to that usually present on interproximal enamel (Preston & Edgar, 2005). Furthermore, the participants had a high caries experience with early loss of deciduous teeth and may have established a more mature and cariogenic plaque.

1.3.3 Surface Free Energy and Surface roughness

Physical and chemical factors significantly influence the attachment biofilms to surfaces. Two main factors that have been implicated in initial adhesion of bacteria are surface free energy (SFE) and surface roughness (Quirynen & Bollen, 1995). Surface free energy (expressed as mJ/m²) is a description of the energy of a substratum like the surface tension of a fluid. The contact angle or relative wettability of a surface is a function of the surface free energies of interacting liquid and solid surfaces and is calculated from the angle formed when the liquid contacts the solid surface (de Jong et al., 1982). Increased contact angle equates to poor wettability. Surfaces with a higher SFE result in an increased amount of biofilm accumulation (Quirynen et al., 1989; Teughels et al., 2006). Bacteria with higher surface free energies colonise higher surface energy materials more readily. However, bacterial cells of the same species may adapt and display an altered SFE depending on the substrata SFE (Weerkamp et al., 1988). The effect of the salivary pellicle on the substratum SFE was studied
by comparing the biofilm formed on a titanium (high SFE) and a Teflon (low SFE) coated implant abutment. The supra- and sub-gingival plaque displayed a less mature plaque in terms of anaerobic organisms on the Teflon surface demonstrating that there is some conversion of physiochemical characteristics (Quirynen & van Steenberghe, 1993).

The influence of surface roughness on SFE has been well documented in the literature. A notable in vivo study examined the effect of varying surface roughnesses on lower and medium SFE polymer strips on plaque formation reporting a four-fold increase in volume of plaque formation on both strips in the rough areas, concluding that the influence of surface roughness is more important in early plaque formation than the SFE (Quirynen et al., 1990). Another in vivo study reported similar findings when examining biofilm formation on various restorative materials (Tanner et al., 2005).

These findings are supported by early electron microscopy studies that demonstrate that biofilm formation begins on enamel and acrylic surface discrepancies and spreads laterally from these areas (Lie, 1978; Nyvad & Fejerskov, 1987). More recent studies investigated the effect that surface roughness has on the nature of early plaque formation and demonstrated that it contributes to a more mature plaque which attaches and grows faster, but it is also more difficult to remove these bacteria and provides an increased area for adhesion (Mierau et al., 1982; Newman, 1974; Quirynen et al., 1990; Quirynen & van Steenberghe, 1989). Furthermore, the initial bacterial attachment and proliferation account for the majority of the microbial constituency in early plaque formation, which further supports the implication that surface roughness is an important element in the development of a potentially cariogenic biofilm (Brecx et al., 1983). Certain bacteria have also been shown to have different attachment profiles to composite resins and orthodontic brackets (Ahn et al., 2002; Steinberg & Eyal, 2002). Streptococcus mutans a known cariogenic species forms a stronger attachment to orthodontic
cements when compared to other streptococcus species (Ahn et al., 2010). The study examined the quantity of *S. mutans* adhesion to three orthodontic adhesives (fluoride-releasing composite, compomer and resin-modified glass ionomer (RMGIC)) and showed that bacteria adhere to all the adhesives significantly more than another initial coloniser *S. sobrinus* (Ahn et al., 2010). Adherence of *S. mutans* to RMGIC was not significantly different to the other adhesives even though RMGIC has greater surface roughness. This demonstrates the important implications surface roughness and orthodontic adhesives have on the initial adhesion of bacteria generally and cariogenic species in particular. Furthermore, it can be proposed that the adhesive remaining around the orthodontic bracket and the resultant increase in surface roughness are crucial predisposing factors in the attachment, accumulation and growth of a pathogenic biofilm in caries-prone individuals.

1.3.4 *Specific, Nonspecific and Ecological plaque hypothesis*

There are a few main hypotheses that have been proposed as to the role of oral biofilm bacteria in the aetiology of enamel demineralisation and other dental diseases. The “Specific Plaque Hypothesis” states that only a few bacterial species are involved in the aetiology of the caries disease process and that treatment should be aimed at targeting these micro-organisms (Loesche, 1976, 1979). On the other hand, the “Non Specific Plaque Hypothesis” proposes that the aetiology of caries is the result of action of all the bacteria species present in the oral biofilm microflora (Theilade, 1986). However, more recently a hybrid hypothesis has been put forward. The “Ecological Plaque Hypothesis” is a mixture of the two former hypotheses and proposes that the carious process is a result of an imbalance in the local environment resulting in more pathogenic species such as mutans streptococci and lactobacilli due to frequent exposure to fermentable carbohydrates and resultant acid production (Figure 2). An important element of this hypothesis is that any species that is acidogenic and acid-tolerant contributes to the disease process (Marsh, 2003).
Figure 2. The “ecological plaque hypothesis” (Marsh, 2003)

Although mutans streptococci and lactobacilli are the most notable and studied species due to their ability to adapt to an acidic environment, other species such as *Streptococcus mitis* add to the demineralisation process and may be responsible for the initial stages of demineralisation when the plaque is devoid of these typical cariogenic species (Brailsford et al., 2001; de Soet et al., 2000; Sansone et al., 1993). The repeated environmental change to lower pH also leads to inhibition of competing bacterial species which allows cariogenic species to adhere and colonise the plaque effectively (de Soet et al., 2000).

1.3.5 Oral Biofilm in Orthodontic patients

It is well known that orthodontic brackets, wires, bands, and elastics with their varying surface roughness and material composition create stagnation areas and make removal of biofilm by tooth brushing and natural cleansing mechanisms of the mouth difficult. Additionally, the area of attachment surfaces for bacteria is significantly increased (Ren et al., 2014). These factors induce specific changes in the local environment such as increased plaque accumulation and maturation which results in a reduced plaque pH in the presence of fermentable carbohydrates resulting in elevated levels of cariogenic species such as *S. mutans*.
and *Lactobacillus* species (Al Mulla et al., 2009; Balenseifen & Madonia, 1970; Chatterjee & Kleinberg, 1979; Mattingly et al., 1983; Mizrahi, 1982).

Bracket systems have evolved significantly since the development of the first edgewise appliance by Edward Angle in 1921 (Wahl, 2005). However, the prevalence of enamel demineralisation around brackets remains high. Therefore, there has been increasing interest as to the attachment of cariogenic and periodontopathic bacteria to these different bracket systems and its clinical significance. However, the studies are inconsistent in their results and are difficult to compare due to the different methodological approaches, different bracket types and manufacturing materials used. Two different types of studies are to be discussed.

*In vitro Studies*

A study examining the adhesion of *S. mutans* and *C. albicans* to metal, ceramic and plastic central incisor brackets *in vitro* found that the adherence was significantly increased to both ceramic and plastic brackets, and a synergistic relationship was evident when both organisms were present (Brusca et al., 2007). The least adhesion was to stainless steel brackets and most bacteria and yeasts were present in the slot of the brackets. No saliva was used in the study and bacterial adherence was compared to attachment to glass tubes. In another study a radioactive label was used to measure *S. mutans* adherence to metal, composite and plastic brackets (Fournier et al., 1998). Lower adherence to stainless steel brackets was confirmed. Saliva coating decreased the adherence to all three materials and although initial adherence was significantly different between brackets, the adherence was not different over time in either saliva-coated or non-saliva-coated brackets. The surface topography of stainless steel, ceramic and plastic brackets is quite varied which almost certainly affects bacterial adhesion (Brusca et al., 2007).
Another study investigated in vitro biofilm formation on seven different types of brackets including self-ligating brackets (van Gastel et al., 2009). They used the plaque and saliva from two orthodontic patients for biofilm formation. Bacteria were isolated from the different bracket types and grown on agar plates to compare the number of aerobes to anaerobes. There was significantly lower bacterial adhesion to stainless steel brackets and ceramic brackets had the highest number of anaerobes. Additionally, self-ligating brackets had greater bacterial attachment when compared with stainless steel brackets ligated with elastomeric modules. Apart from their larger size, scanning electron microscopy (SEM) images reveal irregularities at the welded parts of self-ligating brackets which likely enhance bacterial adhesion (van Gastel et al., 2007).

**In vivo studies**

The distribution of biofilm between orthodontic and non-orthodontic patients has been studied. Molar teeth and the mandibular dentition harbour more plaque in non-orthodontic patients (Furuichi et al., 1992). Contrastingly, in orthodontic patients, biofilm is more prevalent in the maxillary dentition around the lateral incisor and canine brackets. This is thought to be due to the hooks and attachments making this area difficult to clean (Mei et al., 2017). The formation of WSLs also follows this trend, having the highest incidence around the maxillary lateral and canine brackets, molars and mandibular canines (Chapman et al., 2010; Ogaard, 2008; Ren et al., 2014)

Orthodontic bands result in more biofilm formation and a more anaerobic biofilm than bracketed teeth (Diamanti-Kipioti et al., 1987). This may be due to bands being placed near the gingival margin and extension of the biofilm below the gingiva. Placement of bands decreases the resting pH of biofilm formed around bands and increases the S. mitis and S. salivarius counts after only four to five weeks (Balenseifen & Madonia, 1970). Interestingly, other studies have demonstrated there is little mature plaque formation below the gingival margin
following removal of bands after a two-year orthodontic course of treatment suggesting that the host immune response may play a role in bacterial adhesion sub-gingivally (Demling et al., 2009). Another long-term study utilising denaturing gradient gel electrophoresis (DGGE) monitored the changes in the microbial composition of subgingival biofilm for bonded and banded molar teeth during orthodontic treatment and one year following removal of fixed appliances. The study demonstrated significant reduction in the subgingival biofilm population to 40-45% of the pre-treatment plaque. Furthermore, these changes were still evident one year after completion of treatment and were more marked with bonded molar teeth. These findings suggest that bonded molar teeth encourage a more cariogenic microbiota (Ireland et al., 2014)

Self-ligating brackets are publicised by manufacturers to preserve periodontal health as they result in less plaque accumulation due to their lack of elastomeric modules and steel ligatures (Chen et al., 2010). However, in vivo studies show that this is not the case and more anaerobic bacteria are found attached to self-ligating brackets compared to standard stainless steel brackets (van Gastel et al., 2007). Although the bacterial load is increased, the occurrence of WSLs is the same in both groups (Pandis et al., 2008).

The issue of adherence of bacteria to elastomeric modules compared with stainless steel ligatures is contentious. A study utilising plaque sampling (Forsberg et al., 1991) demonstrated that the incidence of bacterial colonisation is higher in brackets ligated with elastomeric modules. However an SEM study on extracted premolar teeth reported no difference in the adherence of bacteria to brackets ligated with either steel ligatures or elastomeric modules (Sukontapatipark et al., 2001). The study also demonstrated that the excess composite around the bracket base was the crucial site for plaque development due to its rough surface topography and presence of voids at the composite-enamel interface (Sukontapatipark et al., 2001). The application of 0.4% stannous fluoride gel had no effect on the populations of S. mutans on both
types of orthodontic arch wire ligation (Bretas et al., 2005). Polymer-coated elastomeric modules which provide less friction, have greater bacterial colonisation compared to elastomeric modules from the same brand (Magno et al., 2008). It has been hypothesised that this is a result of fissures in the polymer created by stretching during placement, thereby facilitating bacterial accumulation (Magno et al., 2008).

1.4 White spot lesion aetiology

Enamel demineralisation adjacent to orthodontic brackets develops due to prolonged biofilm accumulation in this area because of poor oral hygiene and increased use of fermentable carbohydrates (Figure 3 (Ogaard, 2008)). This process undergoes periods of remineralisation and demineralisation. Initially the dissolution begins below the intact surface layer of enamel creating pores between the enamel rods and is referred to as a subsurface lesion. The dissolution of enamel may then continue until the full thickness of enamel (and sometimes dentine) is involved before the hyper mineralised enamel surface layer is lost resulting in a cavitated lesion (i.e. frank enamel caries (Sudjalim et al., 2006)). The intact layer of enamel above the subsurface lesion is thought to occur predominantly due to the physiochemical interactions between the enamel surface and surrounding plaque fluid. The presence of fluoride results in an increased width of this intact surface zone (Fejerskov et al., 2015).

The reported incidence of WSLs varies in the literature amongst non-orthodontic and orthodontic patients. The reason for variability is partly due to the methodology, various WSL scoring indices, different populations and prophylactic measures that are used. Retrospective studies have the advantage of providing a large sample size and power, however, they are prone to bias. On the other hand, cohort studies have the advantage of following patients prospectively and having a control group, however, the sample size is often very small. Keeping all these limitations in mind, the prevalence of WSLs
Figure 3. The Stephan curves from orthodontic patients with good and poor oral hygiene. Placement of fixed appliances lowers the resting pH. An acid attack following consumption of fermentable carbohydrates lowers the pH below the critical pH in patients with poor oral hygiene but not those with effective oral hygiene (Ogaard, 2008).

Pre-treatment ranges from 15% to 70% (Artun & Brobakken, 1986; Gorelick et al., 1982; Lovrov et al., 2007; Ogaard, 1989). Using a scoring system developed for the size and severity of WSLs, a prospective study compared a group of 50 participants at debonding to a control group (Gorelick et al., 1982). The incidence of WSLs doubled with placement of fixed appliances. However, it is important to note that no randomisation of the participants was performed, and the pool of participants was from the authors’ private practice. Another study also compared a group of 60 consecutively treated orthodontic patients with a group of 60 controls (Artun & Brobakken, 1986). More than twice as many of the patients that had received orthodontic treatment had four or more WSLs (33% compared to 12%). A more recent study which examined the records of
400 orthodontic patients reported that 62 % had new or increased WSL following treatment (Enaia et al., 2011).

WSLs can be developed experimentally as early as four weeks (Gorelick et al., 1982; Gwinnett & Ceen, 1979; Ogaard et al., 1988) and as early as three weeks with a heavy sucrose challenge (Von der Fehr et al., 1970). This is the length of time between most orthodontic appointments. The most commonly affected site is the bracket-adhesive-enamel junction, and the most commonly affected teeth are the first molars, upper lateral incisors and canines (Mei et al., 2009; Ogaard, 2008). WSLs often present as a white band around orthodontic brackets on the mesial, distal and gingival surfaces. In severe cases, lesion progression requires early termination of treatment and restorative care unless there is an improvement in oral hygiene and preventive procedures (Ogaard, 2008).

The main mechanism of WSL evolution is the development of a cariogenic environment. There is an increase in the volume of plaque surrounding orthodontic brackets and the resting pH is lower in this plaque compared to plaque in non-orthodontic patients (Chatterjee & Kleinberg, 1979; Gwinnett & Ceen, 1979). Although S. mutans and lactobacilli are associated with caries, and increased levels of these bacteria have been reported in both plaque and saliva of orthodontic patients (Scheie et al., 1984), predicting the development of WSLs according to bacterial counts has been unreliable (Thylstrup & Fejerskov, 1994).

1.4.1 **White spot lesion remineralisation**

Approximately two thirds of WSLs are considered mild (Enaia et al., 2011). 75 % of WSLs improve following removal of orthodontic fixed appliances (Ogaard, 1989). This has been confirmed by scanning electron microscope studies (Artun & Thylstrup, 1989). Progression of these lesions following removal of
orthodontic appliances is between 5-10% (Enaia et al., 2011; van der Veen et al., 2007).

The use of the term “remineralisation” to describe an arrested lesion is contentious. It has been used to describe several phenomena including arrested caries lesions, acid reactions and fluoride interactions with the dental enamel (Thylstrup et al., 1994). Following the publication of an early study (Backer, 1966) which investigated the surface changes of first molars of 90 patients from the age of 7 years till the age of 15 years, it was widely accepted that the clinical appearance of hard, shiny and reversed enamel WSLs was due to salivary repair of the demineralised tissue through redeposition of calcium and phosphate ions from saliva. However, several clinical studies following this initial work demonstrated that the regression of the enamel lesion following removal of the biofilm is in fact due to abrasion and mineral uptake on the surface and not mineral uptake within the body of the lesion (Artun & Thylstrup, 1986; Thylstrup et al., 1994). There is a constant outflow of ions from dentine to the enamel and the pores of WSLs are filled with protein, hence the diffusion of ions from the outside surface into the subsurface lesion is minimal. Therefore, the actual remineralisation in the subsurface lesion is not achieved to any therapeutic degree. The clinical regression of the WSL is a result of surface abrasion and slow redeposition of mineral onto the surface of the incompletely dissolved enamel crystals (Fejerskov & Larsen, 2015).

1.5 pH changes

The characteristic curve that is evident when studying pH changes of dental plaque following the consumption of fermentable carbohydrate was pioneered by Stephan and Miller (Stephan & Miller, 1943). Utilising an antimony electrode, they demonstrated that the plaque pH decreases from 6.5 to 5.0, three minutes following a glucose challenge. The pH returned back to baseline after 40 minutes. This technique has evolved to not only study dental caries and
acidogenicity of foods but also the effects of mouth rinses, chewing gum and other oral hygiene preventive measures (Preston & Edgar, 2005).

Three common methods are used to measure dental plaque pH changes over time; plaque sampling (Carter et al., 1956; Frostell, 1970; Rugg-Gunn et al., 1981), touch (also known as micro-touch method (Kleinberg et al., 1982; Liu et al., 1980; Scheie et al., 1992)), and indwelling electrode telemetric methods (Graf & Muhlemann, 1965; Imfeld, 1977). Although these techniques are able to assess the cariogenicity of foods when used with positive (sucrose) and negative controls (sorbitol) there are differences between the methods when measuring the pH change (van Loveren & Lingstrom, 2015).

The plaque sampling method involves the collection of plaque from teeth with a sterile instrument and mixing it with a pre-determined amount of liquid (often distilled water) and then measuring the pH (Frostell, 1970; Rugg-Gunn et al., 1981). However, this method disrupts the plaque and there is a mix of the inner, outer layers of plaque and saliva. It is useful for giving an average intermittent pH reading of plaque (Preston & Edgar, 2005). The touch method involves the placement of a microelectrode into the interdental space at a point apical to where the teeth contact each other (Kleinberg et al., 1982; Scheie et al., 1992). This method is less disruptive to the plaque and can measure the pH of the deeper layers of plaque; however, saliva contamination is problematic. Furthermore, effective sterilisation is essential when using these electrodes. To overcome the challenges of plaque disruption, indwelling electrodes are placed in removable acrylic plates (Preston & Edgar, 2005). This allows the undisturbed plaque that is formed to be measured continuously in vivo. Although indwelling electrodes record the largest pH fall given their close association with the deepest layers of plaque, they result in unnatural plaque formation on the glass electrode (Preston & Edgar, 2005).
Due to the practical limitations of these methods in vivo and the difficulty in growing a realistic biofilm in vitro, researchers have been investigating other simpler and easier methods to measure the changes in pH of dental plaque. There is literature that has assessed the pH changes of dental plaque surrounding orthodontic brackets (Arab et al., 2016; Balenseifen & Madonia, 1970; Chang et al., 1999; Sengun et al., 2004). Most studies have focused on salivary pH changes, resting plaque pH and changes in pH with preventive measures. There is no identifiable study that has evaluated the influence of adhesive removal methods on plaque pH.

1.6 In situ caries model

Intra-oral cariogenicity (ICT) or in situ models involve the use of an appliance containing a tooth substrate which can be either enamel or dentine to mimic the process of dental caries in vivo (Brudevold et al., 1984; Zero, 1995). Participants remove the devices during eating, drinking and brushing their teeth, and expose the tooth substrate to a carbohydrate challenge that may be experimentally provided or through the participant’s diet. The testing period can range from days to weeks. ICT model systems can be classified based on the design of the appliance, the type of the tooth substrate that is used and the method for assessing demineralisation or remineralisation (Wefel, 1990). These in situ models can be used for plaque pH testing.

The design of the in situ model can involve a removable appliance, single section model or a banded model. The partial denture model is the conventional design used in a number of studies (Phantumvanit et al., 1974). There have been several variations on these appliances including a nylon ring that is bonded to buccal aspect of molar teeth (Robinson et al., 1997), metal and acrylic apparatus housing substrate on the buccal and palatal aspects (Auschill et al., 2004; Benson et al., 1999), to more recent use of thermoplastic splints to improve patient comfort (Burgers et al., 2010; Gu et al., 2012; Hannig et al., 2007). More recent developments involve a double layer thermoplastic splint that houses the
substrate between the two layers (Prada-Lopez et al., 2015). This is claimed to improve stability of the housed substrate, improve patient comfort and allow salivary flow through a hole on each side (Prada-Lopez et al., 2015). Since, all these appliances allow the collection of an intact biofilm and each have their limitations, the choice of appliance will ultimately depend on the study type and objectives.

1.6.1 Bovine Enamel

Several tooth substrates have been used for ICT studies including bovine, porcine and human teeth (Curzon & Hefferren, 2001). Bovine enamel has been used the most extensively as a hard tooth substrate in ICT and erosion studies (Cassiano et al., 2017; Chandler et al., 1990; Hannig et al., 2013; Hertel et al., 2016; Jordao et al., 2016a; Jordao et al., 2016b; Jung et al., 2010; Kensche et al., 2017; Lodi et al., 2015; Martins et al., 2016). However, there are differences in chemical composition between bovine and human teeth. Scanning electron microscopy studies reveals that the bovine enamel crystals are 1.6 times larger than human enamel crystals and there is an increased amount of inter-prismatic substance (Arends & Jongebloed, 1978; Fonseca et al., 2008). Bovine enamel is smoother than human enamel which is more apparent when larger grit size polishers are used (Field et al., 2014). These differences become smaller as the grit size of particles used for polishing decreases (Field et al., 2014). Whilst it has been reported that microhardness values differ between bovine and human teeth (Field et al., 2014), other studies report the values to be very similar (Turssi et al., 2010; White et al., 2010). Although this may result from methodological and preparation differences, it highlights the importance of standardised preparation methods, baseline recordings and the inclusion of controls.

1.6.2 Microhardness

The main objective in ICT studies is determination of the net amount of demineralisation or remineralisation that has taken place during a defined
period. Microhardness testing has been established as a primary method for detection of ion loss through the process of demineralisation in ICT studies (Curzon & Hefferren, 2001). There is a high correlation between the percentage of mineral in a demineralised lesion and the enamel microhardness (Featherstone et al., 1983). Hence cross-sectional microhardness measurements with an indenter is a popular method (Gorton & Featherstone, 2003; Hu & Featherstone, 2005; Paschos et al., 2009; Pascotto et al., 2004).

Nanoindentation involves the use of a diamond-tipped tool that creates an indent in the material being measured. Advances in nanoindentation allow the measurement of the mechanical properties of enamel with an extremely small contact diameter (less than 100nm (He & Swain, 2007b; Oliver & Pharr, 1992)). The load and displacement during the nanoindentation process are recorded by a gauge and the area of indentation is calculated to determine the hardness of the enamel. Furthermore, the elastic modulus can be determined from the load displacement curve that is formed (Fischer-Cripps, 2011).

There are few studies that have examined the subsurface enamel around orthodontic brackets. Pascotto et al. (2004) investigated two adhesives on extracted premolar teeth and made indentations below the brackets, at the composite enamel interface and down the lingual aspect to a depth of 90 µm. Their findings revealed a small area of demineralisation adjacent to the adhesive-enamel interface to a depth of 30 µm. There was a significant difference between the two adhesive groups with composite resin promoting the largest mineral loss (33%) compared to glass ionomer (21%). The microhardness measurements underneath the brackets was similar for both bonding adhesives (Pascotto et al., 2004). An in vitro study examining the effect of demineralisation with six bracket bonding adhesive systems demonstrated that enamel demineralisation developed to a depth of 21 µm and there was significantly less mineral loss when fluoride was incorporated in both composite and RMGIC (Kohda et al., 2012).
There are currently no identifiable studies that have examined the effect of adhesive removal methods on the mechanical properties of the subsurface area of enamel adjacent to orthodontic brackets.
1.7 Objectives

1. To develop an assay protocol to determine plaque pH of an in vitro monospecies biofilm.
2. To develop an intra-oral appliance to hold six bovine enamel discs and generate a clinically relevant biofilm around orthodontic brackets.
3. To determine the optimum time required for in vivo biofilm growth.
4. To compare the effects of three adhesive removal methods around enamel-bonded orthodontic brackets on intra-oral biofilm acidogenicity.
5. To compare the microhardness and elastic modulus changes of bovine enamel for the three adhesive removal methods at the composite-enamel interface and underneath the bracket.
Chapter 2

Methodology
2.1 Research Approach

A prospective study involving participants wearing customised intraoral appliances containing six randomised bovine enamel discs (three on each side) with bonded orthodontic brackets to facilitate intraoral biofilm development.

Research was approved by the University of Otago Human Ethics Committee (Reference number H17/103 (Appendix A)). Consultation was undertaken with the Ngāi Tahu Research Committee in accordance with the Ngāi Tahu/University of Otago Memorandum of Understanding (Appendix A).

The project was undertaken in three parts:

Part I Development of a protocol to measure biofilm pH change after glucose challenge.

Part II A pilot study to develop an intraoral appliance containing bovine discs and to determine the optimum time for in vivo biofilm growth

Part III A prospective randomised experimental study assessing the effects of three methods for removing excess adhesive around orthodontic brackets on in vivo biofilm acidogenicity. The outcome measurements were plaque pH changes following a glucose challenge, surface microhardness, and elastic modulus.
2.2 Part I: Development of a protocol to measure biofilm pH change after glucose challenge

The cariogenic activity of a monoculture biofilm representative of oral bacteria was tested using a pH electrode in vitro.

2.2.1 Enamel discs

Bovine lower incisor teeth were sourced from Silver Fern Farms Finegand Freezing Works (19th September 2017). Freshly extracted bovine incisors, free from macroscopic cracks, caries and staining as assessed by visual examination were allocated for use in Part I, II and III of this study. The crowns were decoronated at the cemento-enamel junction mesiodistally using a high-speed bur with distilled water for irrigation. The pulp was removed and irrigated with 50/50 saline hypochlorite (4% NaHOCl) solution. Each crown was embedded in dental stone (Hinridur, Ivoclar Vivadent, New Zealand) in moulds with the buccal surface sitting above the flat surface of dental stone to allow for polishing. The buccal enamel was polished progressively with 400, 800 and 1200 grit wet/dry sandpaper (Wet/dry carbide paper). Teeth with a lack of enamel were discarded. In total, 81 teeth were prepared in this way.

Nine mm circular discs were sectioned from the polished enamel crowns using a 12 mm diamond core bit (Sutton Tools Pty. Ltd., Australia) and drill press (DP-200B 208mm drill press, Tooline, New Zealand) under distilled water irrigation. A single disc was sectioned from each incisor tooth. To account for variation in enamel composition, a set of at least six discs was obtained from the same mandible. The discs were cleaned with 75% alcohol and placed in a sterile sodium chloride (0.9%) solution (Baxter Healthcare Ltd., Australia) and refrigerated at 4°C with replenishment of NaCl solution at 48-72 h intervals. All the discs were sterilised with gamma irradiation (Gammacell 1000 elite, Best Theratronics, Hercules Taieri Resource Unit, University of Otago) at room temperature (27 °C) with average irradiation dose 3 cGy/min. Irradiation was
for 139 hours to achieve the targeted dose of 25 kGy following the protocol of Viana et al. (2017).

2.2.2 Orthodontic bracket bonding and adhesive removal

Six bovine enamel discs were bonded with orthodontic brackets in the following protocol:

1. An area of approximately 3mm$^2$ was etched for 15 seconds (Scotchbond$^{TM}$ Universal Etchant gel, 3M ESPE), rinsed with distilled water for 20 seconds and lightly air dried.
2. A moisture insensitive primer (Transbond$^{TM}$ MIP, 3M Unitek) was applied to the etched area, lightly air dried and cured for 10 seconds (Flashmax P3 460 4W light curing pen, CMS dental).
3. In order to standardise the quantity of composite, 0.1 ml of orthodontic composite adhesive (Transbond$^{TM}$ XT, 3M Unitek) was delivered via a 1 ml Tuberculin syringe (BD, Singapore) to the base of three right maxillary lateral incisor and three left maxillary lateral incisor brackets (0.018” RMO Alexander brackets, Arthur Hall Orthodontics, New Zealand).
4. Brackets were applied on the prepared surfaces of the bovine enamel discs with equal pressure.
5. The resultant excess adhesive on the six discs was either removed with a universal dental scaler, or left in situ and cured for three seconds (Flashmax P3 460 4W light curing pen, CMS dental) on the mesial, distal, occlusal and gingival aspect. The cured adhesive was removed from two specimens with a tungsten carbide bur (H282K, Komet Dental) to mimic the clinical practice of indirect orthodontic bonding procedures.
2.2.3 Bacterial strain and in vitro culture conditions

*Streptococcus mutans* UA159 was revived from frozen culture maintained in the Department of Oral Sciences (University of Otago). The bacteria were cultured and incubated under anaerobic conditions (85% N₂; 10% H₂; 5% CO₂, in an MG500 workstation, Don Whitley Scientific Ltd.) on Columbia sheep blood agar (Fort Richard Laboratories, New Zealand) for 48 hours. All incubations were at 37°C.

2.2.4 Biofilm culture

Aliquots of an overnight established *S. mutans* culture (100 µL) were added to 9 mL of sterile brain heart infusion broth (BHI (Difco Laboratories, USA)). Filter sterilised (0.45 µm Millex-HV, Merck Millipore Ltd., Ireland) 50 % (w/v) sucrose solution (made up of 50 g sucrose in 100 mL sterile water) was added under the laminar flow cabinet, resulting in a 5% (w/v) sucrose concentration in the starter culture. This was to provide a substrate to encourage extra-cellular polysaccharide production for enhanced biofilm growth. Monoculture biofilms were generated by incubating sterile bovine enamel discs/orthodontic brackets and sterile individual brackets in the *S.mutans* culture (10 ml) at 37°C for 48 hours.

Figure 4: Two glass vials containing brain heart infusion (BHI) supplemented with 5% (w/v) sucrose and enamel bonded orthodontic brackets inoculated with *S.mutans* UA159
2.2.5 Biofilm pH assay determination

Bovine enamel discs bonded with lateral incisor brackets and unbonded lateral incisor brackets were removed from the starter cultures, gently rinsed with distilled water and placed in 15 mL Falcon tubes containing 600 µL of (10% w/v) glucose solution pre-incubated in a water bath at 37°C. Calibration of the Sentron ISFET electrode system was performed according to manufacturer’s instructions prior to each set of measurements (Figure 5). The pH was measured 30 seconds after placing 20 µL of the glucose solution (utilising a pipette tip) onto the electrode surface (CupFET SI series, ISFET electrode system, Sentron Europe BV, Netherlands). The pH was recorded at intervals up to 120 minutes.

Figure 5: pH calibration setup. From left: Sentron ISFET electrode system, pH solution containers 2, 4, 7, 10 & 12.

2.2.6 Determination of Biofilm mass

Biofilm mass was measured by obtaining the initial dry weight of discs with orthodontic brackets and final wet weight (following the glucose challenge) using an analytical balance (Mettler Toledo XSI05 DualRange, Centre of
Excellence, New Zealand). Three subsequent measurements of the final wet weight were recorded after 30 minutes following removal of excess liquid by careful wicking of the liquid from the discs and brackets. However, the repeated weights were quite unstable, therefore biofilm mass was not utilised in the main part of the study.

2.2.7 Enamel dissolution pH assay

Six sterile bovine enamel discs were tested for dissolution of the enamel in glucose solution (10% w/v). Discs were placed in individual Falcon tubes containing 600 µL of glucose solution (10% w/v) in a water bath at 37 ºC. Using a sterile pipette tip, 20 µL of glucose solution (10% w/v) was placed on the electrode surface and the pH was recorded at 0, 5, 10, 20, 40 and 70 minutes.
Part II: Development of an intraoral appliance to hold six bovine discs and to determine the time for optimum in vivo biofilm growth.

Aim: To design an intraoral appliance and determine the protocol for growth of an in vivo multispecies biofilm on bovine enamel discs.

2.3.1 Designing and testing the appliance

The main objectives of the intraoral appliance were to be comfortable for participants to wear continuously for a period of four days to hold the enamel discs and facilitate biofilm growth representative of the clinical situation. For the design, four factors were taken into consideration:

1. Acrylic or thermoplastic design (single or double).
2. Mandibular or Maxillary arch.
3. Palatal or buccal/lingual location.
4. Ease of removal of discs.

Due to the bulky size of the conventional palatal acrylic appliance (Hara et al., 2003), decreased comfort from the brackets on the enamel discs, and palatal biofilm growth that is not representative of the biofilm around orthodontic brackets, a vacuum-formed thermoplastic maxillary design with discs located on the buccal aspect was chosen.

Initially, it was planned to design a single vacuum-formed appliance. An intraoral scan of the teeth was performed (Trioss, 3Shape, Germany) on a volunteer and a 3D model (Figure 6 A) was tested for construction of the appliance (Objet30 Dental Prime, Stratasys, Israel). This would provide a more precise fit for the enamel discs, and thereby allow the discs to be held in place by friction. However, the 1 mm vacuum-formed material (Duran®, Scheu Dental Company, Germany) was ineffective at covering the top aspect of the cylinders on the 3D printed model due to their bulky size which resulted in breakages in
this critical area and loss of discs. A thicker (2mm) vacuum-formed material (Duran®, Scheu Dental Company, Germany) was trialled, however this was firm and uncomfortable intraorally.

Due to the cost and length of time required to print a 3D model, the intra-oral appliances were constructed on dental stone models using 3D printed enamel disc replicas (Objet30, Dental Prime, Stratasys, Israel). Dental impressions were taken and several vacuum-formed thermoplastic appliances were made using 1 mm Duran® acrylic (Scheu Dental Company, Germany). The flexing of the single vacuum-formed thermoplastic material resulted in dislodgement and loss of the discs when the appliance was worn intraorally. Therefore, a double vacuum-formed thermoplastic retainer was trialled (Figure 6 B). An inner layer was fabricated using 1 mm Biobleach™ (1 mm, Scheu Dental, Germany) and the outer layer was constructed using the 1 mm Duran® acrylic.

Six 3D printed discs were used as spacers in the construction of the outer layer of the double-vacuum formed appliance providing a precise fit of the enamel discs bilaterally. A hole was cut on the buccal aspect of the outer layer. The double vacuum-formed thermoplastic appliance was more comfortable due to the soft inner layer. Sticky wax was used to ensure retention of the discs and decrease biofilm accumulation on the edges and inner aspect of the discs which would affect the results of the pH assay (Figure 7).
Figure 6: Development of intra-oral appliance A) 3D printed model of volunteer’s maxillary arch B) Double vacuum-formed appliance containing 3D printed discs (9mm in diameter by 1.8mm depth) secured on the buccal aspect of the inner layer in the canine, premolar and molar area.

2.3.2 Developing protocol for optimum biofilm growth in vivo

To establish the time required for formation of a mature plaque biofilm that would register a pH drop in the assay developed in Part I, two enamel discs containing lateral incisor brackets (RMO) bonded with orthodontic composite adhesive (Transbond™ XT, 3M Unitek) were trialled in a single vacuum-formed retainer that was worn by a volunteer.

The discs were placed in a 15 ml Falcon tube containing 600 µL of (10% w/v) glucose solution following each day of wear. Using a sterile pipette tip, 20 µL of glucose solution (10% w/v) was placed on the electrode surface and the pH measurements were recorded at intervals up to 60 minutes. From this preliminary study it was established that four days of continuous in vivo wear of the appliance would result in a mature biofilm.
2.4  Part III: Effects of three adhesive removal methods around orthodontic brackets on in vivo biofilm development and cariogenicity.

A prospective randomised experimental pilot study to compare the effects of three popular adhesive removal methods around orthodontic brackets on in vivo biofilm acidogenicity.

2.4.1  Participant selection

A convenience sample of eight participants (age range = 25 – 39 years old) was recruited from dental staff and students at the Faculty of Dentistry, University of Otago. Participants were given an information sheet explaining the research goals and what would be involved.

2.4.2  Inclusion Criteria

Participants were included if they were:
1. Over 16 years of age.
2. Self-declared as healthy.
3. Willing to participate and wear a maxillary vacuum-formed appliance continuously for four days.

2.4.3  Exclusion Criteria

Participants were excluded if they:
1. Experienced periodontal disease (greater than 3mm pocked depth).
2. Experienced active dental caries.
3. Used antibiotics in the last three months.
4. Suffered from immune deficiency.
5. Were smokers.
2.4.4 Sample size
Due to the lack of published literature on this research topic, a sample size calculation was not possible, therefore a convenience sample of eight participants was chosen. However, the power was increased through repeated measurements within subjects (six discs per participant).

2.4.5 Study outline

Visit 1
Signed informed consent was obtained. Each participant had an alginate impression taken for construction of their custom-made double vacuum-formed thermoplastic retainer.

Six bovine enamel discs were prepared according to the protocol in Part 1 (Section 2.2.1 and Section 2.2.2).

Visit 2
Each participant was fitted with the custom-made double vacuum-formed retainer containing the six discs (Figure 7). Adjustments were made to the retainer if there were any impinging areas. Discs were randomly assigned to the canine, premolar and molar sites using balanced block randomisation.

2.4.6 Participant instructions
Each participant was given an instruction sheet directing them to continue their normal diet and oral hygiene practices and to remove the appliance for eating and brushing their teeth. They were shown how to clean the inside of the appliance and how to remove the inner layer.

Participants were given a one litre sucrose solution (10% w/v) and three small containers in which to soak the appliance five times a day at least two hours apart for five minutes to promote biofilm growth. Participants were asked to return after 96 hours for assessment.
2.4.7 pH testing

The discs were removed from the appliance and gently rinsed in distilled water (50 mL). For each participant, 600 µL of glucose solution (10% w/v) was placed in six labelled 15 mL Falcon tubes in a 37°C water bath. Three control discs (absence of biofilm) with bonded orthodontic brackets were placed in three labelled 15mL Falcon tubes in the same 37°C water bath. Calibration of the Sentron ISFET electrode system was performed according to manufacturer’s instructions prior to each set of measurements (Figure 5). The discs were distributed in the tubes and the pH of the glucose solution was measured with the electrode system as described in Part I (Section 2.2.5). Briefly, 20 µL of glucose solution was removed and transferred onto the electrode surface. pH readings were recorded at 0, 10, 20, 40, 60 and 120 minutes. The samples were dried by wicking of the liquid from the discs and resting at room temperature (23 °C) for 30 minutes, maintained at 4°C overnight prior to mechanical testing.
2.4.8 Cutting and polishing of enamel samples

Enamel specimens containing the biofilm (each marked with a unique identifier code) were embedded in cold cure epoxy resin (EpoFix Kit, Struers, Denmark) for 24 hours. The specimens were cut in cross-section (Struers A/S, Ballerup, Denmark) and polished (Struers A/S, Ballerup, Denmark) following a modified protocol developed at the University of Otago (Shah Mansouri, 2012). All samples were polished at each step for three minutes in a clockwise and anticlockwise direction (Table I).

Table I. Polishing protocol for enamel samples

<table>
<thead>
<tr>
<th>Step</th>
<th>Force (N)</th>
<th>Abrasive pad</th>
<th>Lubricant</th>
<th>Rotation of polishing disc (rpm)</th>
<th>Cleaning time in ultrasonic bath (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10</td>
<td>Wet/dry carbide paper, grit 1000</td>
<td>Distilled water</td>
<td>150</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>10</td>
<td>Wet/dry carbide paper, grit 2000</td>
<td>Distilled water</td>
<td>150</td>
<td>3</td>
</tr>
<tr>
<td>3.</td>
<td>10</td>
<td>Waterproof silicon carbide paper, grit 4000</td>
<td>Distilled water</td>
<td>150</td>
<td>3</td>
</tr>
<tr>
<td>4.</td>
<td>10</td>
<td>MD-Pan™ Grain size: 1 µm</td>
<td>1 µm diamond suspension</td>
<td>150</td>
<td>3</td>
</tr>
</tbody>
</table>

2.4.9 Nanoindentation system

The hardness (H) and elastic modulus (E) of the cross-section of enamel at two sites of each sample (underneath the bracket and at the composite enamel interface) were determined with an Ultra-Micro-Indentation-System (UMIS, Fischer-Cripps Laboratories, Australia). The UMIS is a nanoindentation tester that applies a loading and unloading force in increments to complete an
indentation cycle. A depth calculation device measures the depth of each indent (Fischer-Cripps, 2011).

An initial contact force indent was applied prior to each indentation to reference the depth calculation device to the enamel sample. A static load of 50mN was applied with n=15 indents 10 µm apart at 10 µm depth from the enamel edge. Compliance was 0.3 nm/mN (Figure 8). A Berkovich indenter is a three-sided pointed pyramid with apex angles of 65.3 degrees which was utilised to produce the indentations with the specifications listed above (Fischer-Cripps, 2011). Post data analysis of the elastic modulus and hardness was performed using IBIS 2 software (Fischer-Cripps Laboratories, Australia).

![Image](image.jpg)

**Figure 8: Screenshot of Nanoindentation settings with the UMIS for testing with a 50mN force (IBIS 2, Fischer-Cripps Laboratories, Australia).**

Each enamel sample was pressed against a circular magnetic base with sticky wax (Model Cement, Metrodent Ltd, Haddenfield, United Kingdom) on a
heating element. The sample and base were allowed to cool for at least 30 minutes prior to testing to reduce the effect of thermally induced changes on the enamel. The cooled samples were mounted on a moveable stage containing a magnet that ensured close contact with the circular base. This stage was controlled by the IBIS 2 software program. The area to be indented was viewed first under a microscope (Fischer-Cripps Laboratories Limited, Kyowa lenses, Sydney, Australia) at 20x to 50x magnification for precise selection of the area to be indented (Figure 9).

The loading and unloading curves were reviewed for each set of indentations. Outliers as determined by the loading/unloading curves, hardness and elastic modulus markers, were excluded. If five or more outliers were present the indentations were repeated to contain at least ten indentations.

Figure 9: Microscopic image (50x) showing 15 indentations, 10 µm apart at 10 µm depth from the enamel edge at the composite-enamel interface.
2.5 Statistical analysis

Raw data was entered and coded in excel (version 16.0.10325.20082, Microsoft Excel, 2016). SPSS was used for all statistical analysis (version 20.0, IBM Corporation, Chicago, Illinois, USA). Data were analysed using mixed-models and, where appropriate, Bonferroni-corrected post-hoc multiple comparisons were run. The mixed model response variables were “pH”, “microhardness” and “elastic modulus”. The variables “site”, “side” and “intervention group” were entered as fixed factors, while the variable “participant” was entered as random term. Type I error was set at 0.05.

2.6 Incentives

Participants were informed they would receive a $100 grocery voucher upon completion of the study.
Chapter 3

Results
3.1 Outline

Section A

Results of the preliminary studies:

1. pH determination of *in vitro* monospecies biofilm.
2. Influence of enamel on pH determination.
3. Determination of optimum intra-oral time for establishment of mature biofilm on bovine enamel discs bonded with orthodontic brackets.

Section B

Results of the main experimental pilot study:

1. Comparison of three adhesive removal methods around enamel-bonded orthodontic brackets on intra-oral biofilm pH following 96 hours *in vivo* exposure.
2. Comparison of the microhardness and elastic modulus changes of three adhesive removal methods at the composite-enamel interface and underneath the bracket following 96 hours *in vivo* exposure.
3.2 Section A

The purpose was to develop and validate a pH assay using an *in vitro* monoculture biofilm representative of gram-positive oral bacteria.

3.2.1 pH determination of *in vitro* monospecies biofilm

Six discs and three brackets on which a *S. mutans* biofilm had formed were removed from the sucrose (5% w/v) and brain heart infusion (BHI), gently irrigated with distilled water and placed in 15 mL Falcon tubes containing 600 µL of glucose solution (10% w/v) in a water bath at 37°C. The pH changes of the glucose solution (10% w/v) were recorded for the discs and brackets (Figure 10). The initial pH of the glucose solution before the discs were added ranged from 6.11 to 6.81. The variation was due to the lack of buffering capacity of the solution. All the discs and brackets had similar decreases below the critical pH of 5.5 over 120 minutes, however the brackets resulted in a higher final pH (Figure 10). All brackets and discs generated a pH decrease to below 5.5 within the first 10 minutes of measurement (Figure 10).
Figure 10: Comparison of pH changes following exposure of six enamel discs and three orthodontic brackets bearing adhered *S. mutans* UA159 monoculture biofilm
3.2.2 Influence of enamel on pH determination

The purpose was to determine the effect of the dissolution of the bovine enamel discs on pH determination in the absence of bacteria.

Six sterile bovine enamel discs were placed in individual 15 mL Falcon tubes containing 600 µL of 10% (w/v) glucose solution in a water bath at 37 °C. The initial starting pH of the glucose solutions before the discs were added ranged from 5.17 to 6.55 (Figure 11). There was a steady increase in the pH of all the discs, except disc one and disc two which both demonstrated an initial drop in pH at 5 minutes followed by an increase at 10, 20, 40 and 70 minutes (Figure 11).

Figure 11. Influence of sterile bovine enamel discs on pH determination
3.2.3 Determination of optimum intra-oral time for establishment of mature biofilm on bovine enamel discs bonded with orthodontic brackets

The purpose of this preliminary experiment was to establish the optimum time required in vivo to develop a multilayered and multispecies biofilm on enamel discs containing bonded orthodontic brackets. Biofilm maturity was determined in the natural environment of the human mouth by pH changes following a glucose challenge ex vivo.

Two discs containing bonded orthodontic brackets were removed daily for four days from an appliance worn by a volunteer and placed in 15 mL Falcon tubes containing 600 µL of glucose solution (10% w/v) and pH was recorded for 60 minutes. The pH changes of the biofilm grown in vivo for up to 96 hours are compared in Figure 12.

After 24 hours of in vivo biofilm growth, both discs registered a rise in pH. Following 48 hours, disc one had a fall to below pH 5.5 whilst disc two had retained a high pH. After 72 hours both discs generated a slightly lower final pH. At 96 hours, both discs displayed similar patterns, an initial rise followed by a steady decrease (Figure 12).
Figure 12. Effect of time on acidogenicity of biofilms developed \textit{in vivo} on enamel discs bonded with orthodontic brackets
3.3 Section B

The effect of three adhesive removal methods around orthodontic brackets on multispecies plaque formation was determined by measurement of pH changes following ex vivo glucose challenge.

3.3.1 Comparison of three adhesive removal methods around enamel-bonded orthodontic brackets on intra-oral biofilm pH following 96 hours in vivo exposure

For all interventions, exposure of the mature biofilm to a glucose challenge (10% w/v) recorded for 120 minutes resulted in a final pH just above the critical pH (Figure 13). There was no significant difference in final pH between the interventions. Compared to all the interventions, the control discs (absence of bacteria) generated a higher final pH following the glucose challenge (p<0.001). The discs that were allocated to the premolar region had the lowest final pH (Figure 14). Their pH was significantly lower than the final pH of discs positioned at the canine sites (Mean difference = 0.39; 95% CI, 0.19-0.66; p=0.006). Discs that were located on the right side of the mouth generated a lower final pH (Mean difference = 0.30; 95% CI, 0.08-0.53; p=0.009) compared to the discs on the left side (Figure 15).
Figure 13: Comparison of three adhesive removal interventions and control (absence of bacteria) following glucose challenge of 96-hour multispecies intraoral biofilm

*Bar graph represents means of 53 individual discs (35 intervention and 18 control discs) and standard error bars. Mixed model analysis using Bonferroni-corrected post-hoc analysis comparing pH and intervention group. The effect of the absence of bacteria on the pH is shown as the control. One disc was lost by a participant during the experimental phase and was excluded from the analysis.
Figure 14: Comparison of pH following glucose challenge of biofilm-bearing bracket assemblies positioned over canine, premolar and molar sites (n=35)

*Mixed model analysis comparing pH, position, intervention and side on all 35 individual discs (for position F=4.3; p=0.022). Bonferroni-corrected post-hoc analysis comparing canine, premolar and molar position. Error bars indicate ±1 standard error.
Figure 15: Comparison of pH following glucose challenge of orthodontic brackets bearing biofilm positioned on the right and left sides of the mouth (n=35)

*Post-hoc analysis with pairwise comparison of pH and side (F=7.74; p=0.009). Error bars indicate ± 1 standard error.
3.3.2 Comparison of the microhardness (H) and elastic modulus (E) changes of three adhesive removal methods at the composite-enamel interface and underneath the bracket following 96 hours in vivo exposure

Nanoindentation was performed on all the in vivo enamel discs using the UMIS nanoindenter with 15 indents at the composite-enamel interface and underneath the bracket. The mean and standard deviation of the E and H were determined according to the force displacement curve produced with the Berkovich indenter at 50mN (Figure 16).

Figure 16: Example of a load displacement (P-h) curve on cross section of enamel underneath the bracket. The y axis displays the load and the x axis the displacement. The results of 15 load displacement curves are shown.

The results of the indentation tests are summarised in Tables 2 to 5.
Table 2. Effect of adhesive removal methods on enamel hardness following 96 hours \textit{in vivo} exposure

<table>
<thead>
<tr>
<th>Intervention</th>
<th>n</th>
<th>Under bracket</th>
<th>Composite interface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bur</td>
<td>11</td>
<td>5.21 ± 0.46*</td>
<td>4.57 ± 0.47</td>
</tr>
<tr>
<td>No-removal</td>
<td>12</td>
<td>4.39 ± 0.45*</td>
<td>4.44 ± 0.46</td>
</tr>
<tr>
<td>Scaler</td>
<td>12</td>
<td>4.32 ± 0.44*</td>
<td>4.71 ± 0.46</td>
</tr>
</tbody>
</table>

*71 and 72 sets of H and E values were obtained each intervention. The mean and Standard Deviation (SD) is presented (Table 2 and 3). Post-hoc analysis utilising a pairwise comparison between the three interventions (p=0.03 Bur and Scaler; p=0.05 Bur and No-removal)

Table 3. Effect of adhesive removal methods on elastic modulus following 96 hours \textit{in vivo} exposure

<table>
<thead>
<tr>
<th>Intervention</th>
<th>n</th>
<th>Under bracket</th>
<th>Composite interface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bur</td>
<td>11</td>
<td>93.30 ± 6.33*</td>
<td>80.83 ± 5.81</td>
</tr>
<tr>
<td>No-removal</td>
<td>12</td>
<td>83.21 ± 6.20</td>
<td>81.08 ± 5.66</td>
</tr>
<tr>
<td>Scaler</td>
<td>12</td>
<td>81.29 ± 6.19*</td>
<td>82.59 ± 5.64</td>
</tr>
</tbody>
</table>

*p=0.04 between Bur and Scaler interventions

There was no significant difference in either the H or E between the three different interventions at the composite-enamel interface. However, there was a difference between the bur and scaler H (p=0.03) and E (p=0.04) of enamel underneath the bracket. The H was also higher for the bur intervention underneath the bracket compared to the scaler (p=0.05).
Table 4. Effect of disc position on enamel hardness following 96 hours *in vivo* exposure

<table>
<thead>
<tr>
<th>Position</th>
<th>n</th>
<th>Under bracket</th>
<th>Composite interface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine</td>
<td>12</td>
<td>4.73 ± 0.45</td>
<td>4.87 ± 0.46</td>
</tr>
<tr>
<td>Premolar</td>
<td>12</td>
<td>5.11 ± 0.45</td>
<td>4.40 ± 0.46</td>
</tr>
<tr>
<td>Molar</td>
<td>11</td>
<td>4.08 ± 0.45</td>
<td>4.45 ± 0.47</td>
</tr>
</tbody>
</table>

SD = Standard Deviation

*p = 0.01 between premolar and molar position

Table 5. Effect of disc position on enamel elastic modulus following 96 hours *in vivo* exposure

<table>
<thead>
<tr>
<th>Position</th>
<th>n</th>
<th>Under bracket</th>
<th>Composite interface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine</td>
<td>12</td>
<td>87.57 ± 6.21</td>
<td>88.48 ± 5.66</td>
</tr>
<tr>
<td>Premolar</td>
<td>12</td>
<td>91.06 ± 6.21</td>
<td>78.92 ± 5.66</td>
</tr>
<tr>
<td>Molar</td>
<td>11</td>
<td>79.16 ± 6.31</td>
<td>77.09 ± 5.78</td>
</tr>
</tbody>
</table>

SD = Standard Deviation

*p = 0.04 between premolar and molar position

There was no difference between either the H and E at the canine, premolar and molar position at the composite-enamel interface. However, the H and E underneath the brackets were higher at the premolar site compared to the molar site (p=0.01 and p=0.04 respectively).
Chapter 4

Discussion and Conclusion
To our knowledge, this is the first study that has investigated the effect of adhesive removal methods on intraoral biofilm pH. The findings indicate that the adhesive removal method has no effect on the formation of a cariogenic biofilm in low risk individuals. A cariogenic biofilm was more correlated with the position of the brackets and side of the mouth.

*In situ* biofilm growth models have been used for assessing the cariogenicity and erosion potential of foods (Chandler et al., 1990; Curzon & Hefferen, 2001; Zero, 1995) and for determination of the structure of dental plaque (Wood et al., 2000). However, few studies have used *in situ* models to study factors relating to microbial haemostasis around orthodontic brackets (Benson et al., 1999; Gameiro et al., 2009). Although there is considerable inter-individual variation and the low sample size diminishes the internal validity, the applicability of this research to the broader population and greater external validity is a strength.

**Preliminary Studies**

Adherence of bacteria to enamel discs containing orthodontic brackets was compared to brackets inoculated with a monoculture of *S. mutans in vitro*. Consistent with previous literature, the current study demonstrated that *S. mutans* adheres to stainless steel brackets in the absence of saliva (Brusca et al., 2007). That the final pH of the enamel discs with bonded orthodontic brackets was lower than brackets alone, supports previous findings of stronger adherence to adhesives by *S. mutans* (Ahn et al., 2010). However, a more likely cause would be the increased surface area of the enamel discs and adhesive. The unpolished dentine side of the enamel discs provided a further roughened surface for bacterial adhesion, which is a critical factor (Quirynen et al., 1990).

The incubation of sterile enamel discs in the glucose solution in the absence of bacteria resulted in a steady increase in the pH. This pattern was also demonstrated with the controls throughout the study. The contact of enamel discs with glucose solution results in the interaction of the water molecules with the HA, causing dissociation of $\text{PO}_4^{3-}$, $\text{Ca}^{2+}$ and $\text{OH}^{-}$ ions from the crystal lattice.
framework, causing a rise in pH (Fejerskov & Larsen, 2015). The variation in pH of the glucose solution prior to placement of discs was an important finding, thought to be due to the absence of buffering capacity of distilled water.

Four days satisfactorily established a mature in situ plaque with cariogenic potential. Initial colonising bacteria undergo rapid proliferation and maturation within 48 to 96 hours (Nyvad & Kilian, 1987) and early plaque formation is influenced by surface roughness, resulting in a more mature and faster-growing plaque (Quirynen et al., 1990; Quirynen & van Steenberghe, 1989). Furthermore, mature plaque is established within four days in the absence of oral hygiene (Furuichi et al., 1992). A notable finding in the present study was the pH drop below critical pH after 48 hours in vivo. This is in agreement with indwelling electrode studies (Imfeld & Lutz, 1980).

The design of the appliance was a modification of previously used intraoral appliances (Gameiro et al., 2009; Gu et al., 2012; Hannig et al., 2007; Prada-Lopez et al., 2015). Although palatal appliances and other in situ mandibular appliances have been used to study demineralisation and remineralisation, significant advantages of the maxillary double-formed thermoplastic appliance were the aesthetics and comfort. Placement of discs on the buccal aspect provided a surface for optimal biofilm growth representative of the in vivo situation. Periodic application of a sucrose solution ex vivo further enhanced biofilm growth whilst avoiding detrimental effects on the participants teeth.

**Experimental study**

We were interested in comparing the effects of three adhesive removal methods on the pH of undisturbed plaque formation around orthodontic brackets bonded to enamel following 96 hours in vivo. The participants were healthy with no signs of active caries or periodontal disease. Eight participants commenced the study, two participants did not complete the study due to illness, therefore our research had a total of six participants.
As with other studies, there was considerable inter-individual variation in \textit{in situ} biofilm development amongst participants (Arweiler et al., 2004; Mei et al., 2017). However, all the biofilms, irrespective of participants, generated final measurements just above the critical pH. There was no difference between adhesive removal methods on plaque metabolism (measured as pH), however, the uninoculated controls always generated a pH significantly higher than the critical pH. This validates the use of this system in measuring plaque pH changes utilising an \textit{in vivo} biofilm growth model. Surface roughness was not measured in this study, which may have provided more information regarding the area of adhesive available for attachment of bacteria. It is also important to note that the recorded pH was of the glucose solution, not the undisturbed plaque directly. pH is the inverse log of the concentration of H$^+$, therefore the H$^+$ would have been diluted in the glucose solution and it would be reasonable to presume that the pH was lower in the biofilm.

The discs fitted at the premolar position generated the lowest final pH, whereas in orthodontic patients the upper lateral and canine teeth generally accumulate more biofilm compared with the premolars (Mei et al., 2017). These findings follow the pattern of non-orthodontic patients where in the absence of orthodontic appliances there is increased biofilm accumulation around the upper molars (Furuichi et al., 1992). It is important to note, that Furuichi \textit{et al.} (1992) utilised plaque index to report on biofilm distribution and did not assess cariogenic potential (eg. pH). A possible explanation for the current finding is the close proximity of the parotid salivary gland duct to the site of placement of the premolar enamel discs (Figure 7). The abundance of salivary enzymes in this area aids in breakdown of dietary starch for bacterial nutrition and contributes to the establishment of biofilm through binding to the tooth surface and oral streptococci (Nikitkova et al., 2013).

The lower final pH on the right side of the mouth was an interesting and unexpected finding. It was first speculated that this was due to the participants being right handed, which affects natural biofilm distribution due to oral hygiene.
practices (Mei et al., 2017). However, the participants in the present study were instructed not to brush their discs. Another possible explanation relates to the laboratory methodology, in which the right-hand discs were tested in the glucose solution prior to the left discs for standardisation purposes. It is unlikely that this would have caused a significant alteration in the biofilm acidogenicity as all the discs were maintained in distilled water at 37°C and the delay was only two minutes.

**Enamel Microhardness and Elastic modulus**

Cross-sectional microhardness is widely used to study mineral changes in enamel (Benson et al., 1999; Gameiro et al., 2009; Gorton & Featherstone, 2003; Pascotto et al., 2004). However the measurements for both H and E vary considerably depending on whether the indentation tip is closer to the outer aspect of enamel or near the enamel-dentine junction (He & Swain, 2007a). Cross-sectional microhardness of bovine enamel (4.65 GPa) is slightly higher than human enamel (4.32 GPa (White et al., 2010)) and the values of 4.44-4.71 GPa at the enamel-composite surface reported here concur with the previous findings. The variations at both the composite interface and underneath the bracket are due to the different thickness of enamel that result from the polishing process to achieve a flat enamel disc. Some teeth were more rounded and polishing resulted in a thinner layer of enamel, whilst others had a relatively flat profile, and therefore had a thicker layer of enamel. The enamel specimens were polished flat to reduce confounding variables, future studies should consider using unpolished enamel for demineralisation experiments to reduce the variation in microhardness measurements (Fejerskov & Larsen, 2015; Mullan et al., 2018a).

There was no significant difference in microhardness between adhesive removal methods at the composite-enamel interface. Fluoride increases the enamel hardness within a depth of 20 µm (Wilson & Love, 1995), which may explain the increase in H values for the scaler and no-removal interventions compared to the internal control (underneath the bracket). This finding could also be attributed to the effect of acid etching resulting in mineral loss underneath the bracket, and
therefore lower H values (O’Reilly & Featherstone, 1987; Pascotto et al., 2004). The bur removal intervention resulted in a slight decrease of H at the composite-enamel interface compare to the bur removal internal control (underneath the bracket), and this may be attributed to loss of enamel through mechanical damage by the tungsten carbide bur.

The difference in H and E underneath the bracket between the bur and scaler interventions and between premolar and molar positions is difficult to explain, but could be due to the acid etching procedure during placement of the adhesive which can result in 3 to 8% mineral loss underneath the bracket (O’Reilly & Featherstone, 1987; Pascotto et al., 2004). The enamel specimens were prepared in sets of three which resulted in the etch being in contact with the second and third enamel specimens for slightly longer. Bovine enamel erodes 30% faster than human enamel (White et al., 2010), and this may be an indication to adjust the etching time in future studies.

The significant difference in final pH measured at the premolar position compared to the canine position was not evident with the H values when comparing the bracket positions. Although not significant, the E and H had the largest difference at the premolar position. The difference in H and E between the premolar and molar position at the internal control (underneath the bracket) is difficult to explain. Randomisation of disc placement was performed to eliminate bias, but it is possible that the enamel was thicker simply by chance in the premolar sites.
Future Directions

This study builds on our understanding of the acidogenicity of biofilms around orthodontic brackets *in vivo*. The unwanted side effects of WSLs are still unquestionably prevalent (Ren et al., 2014), however, research on adhesive removal methods is lacking. To further develop our clinical based guidelines for removal of adhesive around orthodontic brackets, future research is needed to address the questions raised by this study.

A clinically relevant finding in this study was the low pH generated by the biofilm in very low caries risk adult participants across the three intervention groups. This suggests removal of excess adhesive in low caries risk individuals may not be such a critical factor in the formation of a cariogenic biofilm. However, what remains to be determined is the effect that these established adhesive removal methods have on high caries risk individuals, particularly the teenage population. Future research should address this with well-designed randomised clinical trials.

A short duration *in vivo* exposure was chosen for this study to increase compliance. There was little evidence of demineralisation when comparing the adhesive removal methods using nanoindentation, this is likely attributed to the short duration of intra-oral time in low caries risk participants. Future studies should consider a more sensitive tool such as contact profilometry to compare enamel surface changes adjacent to the composite-adhesive interface for short duration *in vivo* experiments (Mullan et al., 2018b). However, this would require the removal of the bracket whilst leaving the adhesive intact, which remains a technical challenge.

The strength of an *in situ* biofilm growth model using a tooth substrate to assess the demineralisation and remineralisation around orthodontic brackets, is the ability to expose the substrate to natural environmental conditions and measure mineral loss without extraction of teeth. This study showed that a representative intra-oral biofilm may be formed on bovine enamel discs to which orthodontic brackets are bonded, this *in situ* model may further be utilised to test the efficacy
of antimicrobial agents incorporated into orthodontic materials and other developments in orthodontic adhesive technology.

Although not significant, there was additional mineral loss at the enamel-composite interface with the bur adhesive removal method compared to the scaler and no removal methods. Presumably due to mechanical damage on the enamel from the tungsten carbide bur. With the increasing popularity of the indirect bonding technique in application of fixed appliances and the use of burs for removal of excess adhesive, further research needs to address the effect this has on the loss of the outer layer of enamel, the native resistance of enamel to demineralisation (Mullan et al., 2018a).

The low sample size of this study is a limitation. There is a need to undertake more costly, time consuming clinical trials involving a larger number of participants to further understand the effects of adhesive removal methods on the acidogenicity of biofilms and resultant WSLs in high risk patients. The effect of ligation methods and archwire combinations was not incorporated into this pilot study and future research should include this to provide a more clinically relevant study model.
Conclusion

This study devised and validated an *in situ* biofilm growth model for assessing acidogenicity of biofilms formed on enamel discs to which brackets were bonded. The technique was then applied to compare the potential cariogenicity of biofilms formed around brackets following removal of adhesive by three established methods.

The trial necessarily involved healthy adults of very low caries risk and, despite promoting a cariogenic biofilm by periodic exposure to sucrose, there was little evidence of demineralisation even with the relatively low pH generated by the biofilms.

1. There was no difference in adhesive removal method on the acidogenicity of the plaque around the orthodontic brackets. However, an acidogenic plaque established within 96 hours *in vivo* in low caries risk individuals.

2. The premolar sites generated lower final pH measurements compared to the molar and canine sites, suggesting that the position, natural plaque formation and salivary duct influence biofilm acidogenicity. The discs positioned on the right side generated lower final pH values than those on the left. This was thought to be due to natural plaque formation and laboratory methodology.

3. There was no difference in enamel microhardness and elastic modulus measurements following the three adhesive removal methods, although there was a difference at the internal control (underneath the bracket) which may be attributed to the etching and preparation of the discs.
References


Appendix A

Ethical Approval

Ngāi Tahu consultation
Dear Dr Antoun,

I am writing to let you know that, at its recent meeting, the Ethics Committee considered your proposal entitled "Effects of different adhesive removal methods on bacterial colonisation on in vivo orthodontic bracket model".

As a result of that consideration, the current status of your proposal is: Approved

For your future reference, the Ethics Committee's reference code for this project is: H17/103.

The standard conditions of approval for all human research projects reviewed and approved by the Committee are the following:

Conduct the research project strictly in accordance with the research proposal submitted and granted ethics approval, including any amendments required to be made to the proposal by the Human Research Ethics Committee.

Inform the Human Research Ethics Committee immediately of anything which may warrant review of ethics approval of the research project, including: serious or unexpected adverse effects on participants; unforeseen events that might affect continued ethical acceptability of the project; and a written report about these matters must be submitted to the Academic Committees Office by no later than the next working day after recognition of an adverse occurrence/event. Please note that in cases of adverse events an incident report should also be made to the Health and Safety Office:

http://www.otago.ac.nz/healthandsafety/index.html

Advise the Committee in writing as soon as practicable if the research project is discontinued.

Make no change to the project as approved in its entirety by the Committee, including any wording in any document approved as part of the project, without prior written approval of the Committee for any change. If you are applying for an amendment to your approved research, please email your request to the Academic Committees Office:
Approval is for up to three years from the date of this letter. If this project has not been completed within three years from the date of this letter, re-approval or an extension of approval must be requested. If the nature, consent, location, procedures or personnel of your approved application change, please advise me in writing.

The Human Ethics Committee (Health) asks for a Final Report to be provided upon completion of the study. The Final Report template can be found on the Human Ethics Web Page http://www.otago.ac.nz/council/committees/committees/HumanEthic/Committees.html

Yours sincerely,

[Signature]

Mr Gary Witte
Manager, Academic Committees
Tel: 479 5256
Email: gary.witte@otago.ac.nz

c.c. Professor W M Thomson  Department of Oral Sciences
Wednesday, 07 June 2017.

Dr Joseph Auton,
Faculty of Dentistry - Department of Oral Science,
DUNEDIN

Tēnā Koe Dr Joseph Auton.

**Effects of different adhesive removal methods on bacterial colonization in an in vivo orthodontic bracket model**

The Ngāi Tahu Research Consultation Committee (the committee) met on Tuesday, 06 June 2017 to discuss your research proposition.

By way of introduction, this response from the Committee is provided as part of the Memorandum of Understanding between Te Rūnanga o Ngāi Tahu and the University. In the statement of principles of the memorandum it states "Ngāi Tahu acknowledges that the consultation process outlined in this policy provides no power of veto by Ngāi Tahu to research undertaken at the University of Otago". As such, this response is not "approval" or "mandate" for the research, rather it is a mandated response from a Ngāi Tahu appointed committee. This process is part of a number of requirements for researchers to undertake and does not cover other issues relating to ethics, including methodology they are separate requirements with other committees, for example the Human Ethics Committee, etc.

Within the context of the Policy for Research Consultation with Māori, the Committee has based consultation on that defined by Justice McGeachan:

"Consultation does not mean negotiation or agreement. It means: setting out a proposal not fully decided upon, adequately informing a party about relevant information upon which the proposal is based; listening to what the other has to say with an open mind (in that there is room to be persuaded against the proposal); undertaking that task in a genuine and not cosmetic manner. Reaching a decision that may or may not alter the original proposal."

The Committee considers the research to be of importance to Māori health.

The Committee notes and comments that ethnicity data is to be collected as part of the research project and recommends the use of the questions on self-identified ethnicity and descent, these questions are contained in the latest census.

The Committee notes and comments the undertaking to disseminate the findings to relevant Māori health organisations; for example the National Māori Organisation for Dental Health, Oranga Tino and further suggest dissemination to Professor John Broughton and Malcolm Dacker, who are involved in Māori Dental Health, University of Otago.
We wish you every success in your research.

This letter of suggestion, recommendation and advice is current for an 18 month period from Tuesday, 06 June 2017 to 6 December 2018.

Nāhaku noa, nā

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Appendix B

Information Sheets
Effects of different adhesive removal methods on bacterial colonisation on in vivo orthodontic bracket model

Thank you for showing an interest in this project. Please read this information sheet carefully. Take time to consider and, if you wish, talk with relatives or friends, before deciding whether or not to participate.

If you decide to participate we thank you. If you decide not to take part there will be no disadvantage to you and we thank you for considering our request.

What is the aim of this research project?

Braces make it more difficult to brush teeth properly and can increase the buildup of plaque (which contains bad dental bugs) in these hard-to-brush areas. A large amount of plaque can lead to problems like white spots on teeth, decay, bleeding gums and bad breath.

The area on the tooth that is the most vulnerable is where the glue from the bracket contacts the tooth. When a bracket is placed on a tooth the glue squeezes out underneath the bracket onto the tooth surface. This extra glue is removed in different ways by the Orthodontist.

We are looking at how the different ways of removing this extra glue affects the growth of the bad dental bugs. This will help us find out a good way of removing this glue from around orthodontic brackets.

This study is being done by Ana Low, who is a dentist studying to be an Orthodontist at the School of Dentistry, University of Otago.

Who are we looking for?

We are looking for healthy participants over the age of 16 years who have well aligned upper teeth and are willing to wear a plastic plate covering their upper teeth, containing six bovine enamel discs with brackets attached to the plate for four full days.
We cannot include people who:

- are smokers (including social)
- have gum disease
- have braces
- have crooked teeth
- have a health condition (i.e. diabetes)
- have taken a course of antibiotics in the last 3 months

If you participate, what will you be asked to do?

First visit

On your first visit, Ana Low will take a mould or scan of your upper teeth. This will be used to make a double layered custom made plastic plate that only covers your upper teeth.

Second visit

The plastic plate will be delivered and tried in your mouth. If there are any areas that are not comfortable these will be adjusted. There will be six nine mm bovine enamel discs on the plate, three on each side over your molar, premolar and canine region (these are on the cheek side). Brackets will be bonded on these discs using the standard procedure for placing brackets for orthodontic patients and the discs will be secured with sticky wax.

You will be asked to wear your plate full time for four days (including nights). The plate can be removed for eating and brushing your teeth. You will be given a container to store your plate when you are not wearing it. We ask that you store it in a warm place during this time.

You will be required to immerse the plate in a sugar solution (10% sucrose) for 5 minutes five times a day at no less than hourly periods betweenimmersions. Following immersion gently rinse the plate with warm tap water.

Third and final visit

You will return to the Orthodontic department after 96 hours for tests to be carried out on the discs. The discs will be placed in a 10% glucose solution and pH measurements taken at different time intervals. We ask that you do not eat anything 2 hours prior to coming for your visit.

What will we do?

- Measure the pH of the glucose solution
- See how much bacteria has grown on the disc by weighing it after four days
- Measure how much softening of the enamel surface has occurred at the bracket tooth interface by doing mechanical stress tests on the discs after four days
Is it uncomfortable and is it safe?

The plastic plates used are very comfortable and are the same plates that we use for our orthodontic retainers. The brackets are also the same orthodontic brackets that we use for all our patients at the department of Orthodontics. Patients report these to be very comfortable, and as there are no wires and elastics very little discomfort for this short period is anticipated. However, wax will be supplied for any areas that may rub on your cheek.

The enamel discs have been cut from bovine (cow) enamel sourced from Silver Fern Farms. Bovine enamel has been used in this way both in New Zealand and around the world for a number of years, including at the University of Otago. They have undergone a very rigorous preparation, cutting and sterilisation procedure through the Otago University Medical School and Faculty of Dentistry utilising top of the range medical grade equipment and sterilisation.

What information and data will be collected?

Age, gender, amount of bugs in plaque, pH changes and enamel surface hardness changes.

Privacy protection

Information will only be used by Ana Low and kept completely private. Data will be stored in a password-protected database and only Ana Low and her supervisors (Dr. Joseph Antoun, Dr Li Mei, Associate Professor Geoffrey Tompkins and Professor Mauro Farella) will have access to the collected information. Furthermore, all the collected information will be destroyed, ten years after the research is finished.

Data will be used to:

Compare plaque pH, enamel hardness changes and the amount of bad bugs with other participants in the study. We may use the results of the study for other similar research and publish them in healthcare journals or use them in healthcare presentations.

If you agree to participate, can you withdraw later?

Participants can withdraw from the study at any time and without any disadvantages of any kind.

Voucher

A $100 New World Voucher will be given as a thank you for participation on your last visit.

Any questions?

If you have any questions now or in the future, please feel free to contact either:

Ana Low 0272566599  ana.low@postgrad.otago.ac.nz
This study has been approved by the University of Otago Human Ethics Committee (Health). If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (phone +64 3 479 8256 or email gary.witte@otago.ac.nz). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.