12-2011

Bacterial adherence of Streptococcus mutans and Lactobacillus acidophilus on poly-methyl methacrylate and thermoplastic polypropene used in orthodontic retention

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BACTERIAL ADHERENCE OF *STREPTOCOCCUS MUTANS* AND *LACTOBACILLUS ACIDOPHILUS* ON POLY-METHYL METHACRYLATE AND THERMOPLASTIC POLYPROPENE USED IN ORTHODONTIC RETENTION

by

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2008

A thesis submitted in partial fulfillment
of the requirements for the

Master of Oral Biology

Department of Orthodontics
School of Dental Medicine
The Graduate College

University of Nevada, Las Vegas
December 2011

UNIVERSITY OF NEVADA LAS VEGAS

THE GRADUATE COLLEGE

We recommend the thesis prepared under our supervision by

Lindsay Ann Pfeffer

entitled

Bacterial Adherence of Streptococcus Mutans and Lactobacillus Acidophilus on Poly-Methyl Methacrylate and Thermoplastic Polypropene Used in Orthodontic Retention

be accepted in partial fulfillment of the requirements for the degree of

Master of Oral Biology
Department of Orthodontics

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December 2011

ABSTRACT

Bacterial Adherence of *Streptococcus mutans* and *Lactobacillus acidophilus* on Poly-methyl methacrylate and a Thermoplastic Polypropene used in Orthodontic Retention

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Retention is required in the majority of orthodontic patients throughout the remainder of their life. The two primary removable appliances are known as the traditional Hawley retainer or the vacuum formed retainer. These appliances were developed to maintain the position of the dentition without sacrificing oral health. The orthodontic population is at a higher risk for caries due to plaque accumulation from poor diet, suboptimal oral hygiene and often lack of motivation. These two retainers occupy different niches and are comprised of different materials; therefore the retainers' effect on oral health could be very different. An understanding of which bacteria and to what extent the bacteria adhere to these two retention appliance materials could ultimately provide clinicians with another factor to consider when choosing a specific retainer.

Two common caries bacteria, *Streptococcus mutans* and *Lactobacillus acidophilus,* were chosen to study their adherence properties on two common retention materials; polymethyl methacrylate in the traditional Hawley retainer and a thermoplastic polymer made of polypropene in the vacuum formed

retainer. Bacterial adhesion tests on both materials were run either with or without prior coating in saliva and the number of adhered bacteria was determined by both directly counting colony-forming units of bacteria swabbed from the materials and by inference from total metabolic activity of the adhered bacteria as determined by incubation with the tetrazolium dye,sodium2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt)(XTT) reagent.

Culture analysis from adhesion testing determined through colony forming units, showed an increased adherence of both bacteria to polymethyl methacrylate compared to Polypropene. This was reflected in a 3-fold increase for *Lactobacillus acidophilus* and 7-fold increase for *Streptococcus mutans* on the polymethyl methacrylate. Bacterial adhesion testing performed using the metabolic XTT proliferation assay also demonstrated increased adhesion on polymethyl methacrylate. Bacterial adhesion to polypropene was decreased by 30% for *Lactobacillus acidophilus* and 27% for *Streptococcus mutans* compared to polymethyl methacrylate. XTT assay also indicated that prior coating of materials to saliva had little effect on the extent of bacterial adhesion.

In conclusion, bacterial adherence is increased for polymethyl methacrylate when compared to polypropene, regardless of the assay technique used to determine the number of adhered bacteria. Further research needs to be conducted to determine if increased adherence to polymethyl methacrylate is significant enough to influence choices for orthodontic retention.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank the members of my committee for providing both your time and skill to this project. I would like to thank Dr. Lemon, my committee chair and Dr. Martin, my orthodontic faculty for your continuous support with this research, throughout all its ups and downs. I would like to thank Dr. Cruz for devoting much of her time to me and the original design of this study. I would like to thank Dr. Kingsley for help both in the lab and with all the statistical analysis. Additionally I would like to thank Dr. Howard, for her relentless efforts with this project and providing me with everything necessary to make this project a success. Without her this project would not have been possible.

Most importantly I would also like to thank all those individuals who have helped me throughout my life and have shaped me to be the person that I strive to be. Without them I am nothing. I owe great thanks to my family, friends and past teachers for supporting me throughout all of life's endeavors. I especially would like to thank my mother, Buteena and Daniel Riley for helping me on a day-to-day basis with the complexities of life and loving me unconditionally.

And finally, I would like to dedicate this to my father, the late Dr. Pfeffer who never knew I would follow his footsteps so closely. His ability to embrace his profession and yet touch the hearts of his patients, has left not only an indelible mark on me, but is something I hope to continue throughout my own life.

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CHAPTER 1

INTRODUCTION

All orthodontic patients wear retainers for an extended amount of time post treatment, some indefinitely. This retainer can either be fixed or removable, depending on both the needs and preferences of the patient and orthodontist. The two main types of removable retainers most often used in orthodontics are the traditional Hawley retainer and the Thermoformed retainer. Although both retainers serve as reliable methods of retention, their physiochemical properties and relative location within the oral cavity, present them with unique biological differences.

The Hawley retainer is a tissue born retainer made of polymethyl methacrylate (PMMA), which rests on the gingiva. The retainer consists of a metal bar and clasps for retention of both the teeth and appliance. It is most often used when settling or fine adjustments in the dentition are being attempted (Lindauer,S.J. 1998).

The thermoformed or vacuum formed retainer (VFR) is a tooth borne removable retainer that covers the entire surface of all teeth being retained. It is a single layer of thermoplastic polymer made up of polypropene (PP). Most often the VFR is used for retention when no adjustments are needed in the dentition or the patient desires a more esthetic, less cumbersome form of retention (Sheridan,J.J. 1993).

In addition to their obvious difference in design and implications for retention, these retainers differ greatly in cost, durability and skill required. A majority of research comparing these two retainers has investigated their ability to retain the dentition and compared overall satisfaction of the appliance by both the clinician and patient. Little research has been done to investigate these retainers' close relationship with bacteria in a caries prone population, such as a teenage orthodontic patient.

The etiology of caries, gingivitis and periodontitis is largely due to the bacterial plaque, and certain bacteria exist at high rates in the oral cavity of many patients, especially orthodontic patients. Biofilms formed from early plaque bacteria increase as the patients' oral hygiene and diet worsen. Dental plaques can thicken, thus providing optimal environmental conditions for otherwise transient bacteria. This environment further allows bacteria to successfully survive and proliferate. Due to the specific and ecological plaque hypotheses, we know that certain bacteria inhabit specific locations in the oral cavity, and the bacteria's ability to cause disease is influenced by environmental conditions that enable them to flourish (Marsh, P.D. 1994). Based on the availability of nutrients, specific bacteria can render their own individual implications to oral health (Kleinberg, I. 2002). Therefore, any appliance that has the ability to attract and collect plaque can cause or exacerbate disease.

An oral ecosystem that was often kept in balance through the multiple roles of saliva, can suddenly see shifts of bacterial populations and pH often leading to caries. Since one retainer is tooth borne, while the other is tissue

borne it is plausible differences in the amount of bacterial adhesion and accumulation may exist between these two retainer materials based on their very design. Their different placements and retentive nature, could create a possible niche favoring bacterial invasion. This would be in addition to any differences that might be inherent to the materials, such as physiochemical properties altering adhesion forces. Physiochemical factors such as surface free energy, hydrophobicity, and porosity could affect one material's bacterial adherence properties compared to the other (Papaioannou, W. 2007, Quiryen, M. 1995)

To date, limited studies have focused on bacterial adherence in VFR's and how VFR compares with bacterial adherence to PMMA acrylic. This lack of research on VFR is troubling, despite its ability to encase the tooth and essentially create its own microenvironment conductive to bacterial growth and high acidity (Botha, S.J. 1993). Only one case report documents severe tooth demineralization in a patient after extended VFR wear (Birdsall, J. 2008). Whether the acidic environment necessary for severe demineralization was created or facilitated by the adherence of bacteria to VFR material is still unknown. Based on this report, it is important to evaluate possible differences in bacterial adhesion of VFR material compared to that of the Hawley retainer because it could affect oral health thereby altering orthodontic choices for retention.

Past research of bacterial adherence to PMMA and the nature of orthodontic oral flora, allowed a foundation for testing the adhesion of PMMA in comparison to VFR. The microbial species *Streptococcus mutans* (SM) and

Lactobacillus acidophilus (LA) were selected for testing due to their pivotal role in caries as well as their predominance in caries research (Birdsall,J. 2008).

Streptococcal species are a major constituent of dental plaque and are believed to initiate caries. This early colonizer adheres to salivary proteins on the tooth's surface. In addition, an enormous amount of research on the adherence of bacteria to orthodontic appliances has been conducted. The majority of this research focused on bacterial adhesion to elastics or metal brackets (Ahn,S. 2005; Kitada,K. 2009). Studies on patients with fixed orthodontic appliances found increased numbers of streptococci in supragingival plaque (Leung,N.M. 2006). Large numbers of SM have been associated with increased dental caries and infection. This bacterium metabolizes the sugar in our diets, and the resulting acid lowers the oral pH. This acidic environment is optimal for SM growth and often results in caries. Individuals with a diet high in carbohydrates and poor oral hygiene are at greatest risk for SM induced caries, most often mirroring an orthodontic patient (Sari,E. 2007).

Lactobacillus acidophilus like SM is considered normal flora, with increasing levels often indicating advancing caries (Loesche,W.J. 1986). They are primarily secondary invaders that are abundant in deep cavities where acidity is highest (Botha,S.J. 1993; Nyvad,B. 1993). Although it needs a pioneer organism to attach, this opportunistic organism often takes advantage of environmental conditions and the source of nutrients most often seen with orthodontic appliances (Botha,S.J. 1993). LA favor the anaerobic conditions

often seen in orthodontic patients, and with SM are known as common caries indicators (Ollila,P.S. 2008; van Houte,J. 1994).

If differences in adherence exist for either of these species, between the PMMA and VFR material, it is possible that certain retainers should not be prescribed to those patients with high caries rates. If a certain type of retainer is required for other orthodontic retention reasons and this retainer's material results in a high microbial adherence, it may indicate to clinicians an increased need for a disinfection protocol with adjuncts such as fluoride. The goal of this research is to determine if either of these two caries promoting pathogens differentially adheres to each of these retainer materials and to what extent. Clinicians can later extend this knowledge to its potential clinical implications for better orthodontic retainer selection, effective protocol and possibly improved oral health.

CHAPTER 2

REVIEW OF RELATED LITERATURE

2.1 Caries

Caries or dental decay is one of the most common and costly diseases we face today. According to the World Health Organization, caries is present in over 60 to 90 percent of school children (Marsh,P.D. 2005). Caries risk varies with age, socioeconomic status and ethnicity according to the individuals own immune response (Marcotte,H. 1998). Caries is often thought to be preventable due to caries relationship with plaque abundance, therefore much of the therapeutic focus has been on chemical and physical plaque removal (Wilson,M. 1989). However, gingival changes are most often transient, resulting in no permanent damage to the hard and soft tissues (Atack,N.E. 1996). Due to the overwhelming amount of individuals with caries, numerous models have been developed to better understand the caries phenomenon. The caries model developed by Keyes, relates the host's diet with relative amounts of plaque bacteria (Forssten S.D et al, 2010). This model portrays the importance of lifestyle and behavior in predicting caries (Forssten S.D et al, 2010; ten Cate,J.M. 2009). Here the host contributes to caries based on their relative diet, saliva, crevicular fluid and immune response. From this point forward the bacterial composition will shift with differing stages and ultimately reflect the severity of disease (Cohen,B. 1980; Loesche,W.J. 1979). Although indigenous bacteria are often compatible with the host, even in high numbers, often transient or exogenous bacteria can cause disease. It is this imbalance of normal flora that can become pathogenic

and led scientists to adopt the specific plaque hypothesis; now more recently replaced by the ecological plaque hypothesis (Marcotte,H. 1998). This change in hypothesis reflected that bacteria prognostic for disease, were in fact more, environment driven. Bacteria inhabit saliva and hard and soft tissues, but the host's diet contributes to low pH. This low pH increases acidogenic bacteria, aggravating the condition (Tanner,J. 2000). This revised hypothesis, accounts for the fact that without carious conditions, cariogenic bacteria are insignificant (Marsh,P.D. 2004).

2.2 Plaque's Role

Plaque, the primary etiological agent in caries, is a complex yet stable ecosystem developed on the surface of the tooth (Atack,N.E. 1996; Gibbons,R.J. 1973; Socransky,S.S. 1971). It matures sequentially from inter and intra species interactions within the host, allowing bacteria to adhere and colonize the tooth's surface (Socransky,S.S. 1971). These bacteria form what is known as a biofilm, varying between persons and its location in the oral cavity. Most often this biofilm is harmless, but when bacterial and environmental conditions prevent equilibrium, caries and periodontal disease may occur. Biofilms are formed in four main phases. Phase one involves Brownian movement and bacterial chemotaxis, in which bacteria are transported to a given surface (Quirynen,M. 1995). Phase two involves interaction between that surface and the bacteria (Gibbons,R.J. 1973; Quirynen,M. 1995). The resulting initial adhesion is due to Van der Waals forces and electrostatic attraction, which often is reflected as the

surface free energy of the material (Bollen,C.M. 1997). Phase three represents a firmer attachment with specific ionic, covalent and hydrogen bonds bridging bacteria to the surface through specific extracellular proteins. In this phase not only surface tension, but hydrophobicity and bacterial affinity for salivary proteins has an effect on adhesion (Wilson,M. 1989; Busscher,H.J. 1984). Bacteria now attached, begin to grow and often overcome the shear forces of saliva and mastication (Gibbons,R.J. 1973). This is the final phase, in which different bacteria proliferate and colonize the surface by co-adhesion and coaggregation (Quirynen,M. 1995). As plaque levels increase, and species diversity evolves, the plaque becomes harder to remove and more pathogenic in nature (Leung,N.M. 2006). This biofilm, which was once reversible, under the right conditions, quickly becomes more established. This enhanced communication between bacteria is now considered irreversible. Coaggregation and coadherence between bacterial cells coordinate a firm community, which with time becomes more gram-negative and anaerobic, leading to enamel dissolution (Kolenbrander,P.E. 2000; Kolenbrander,P.E. 2002; Nyvad,B. 1993).

2.3 Saliva's Contribution

Saliva is made up of 98% proteins, and contributes to the equilibrium that keeps the oral cavity in a disease-free state (Castro,P. 2006; Vitorino,R. 2006). Through the acquired pellicle, salivary composition becomes the substrate for bacterial inhabitation (Vitorino,R. 2006). In addition to proteins, saliva contains carbohydrates and lipids which can vary with a host's diet and oral hygiene

(Hahnel,S. 2008). Social, psychological, biological, genetic and environmental factors can all contribute to a person's salivary state (Tenovuo,J. 1997). Saliva's components have the ability to both compromise and protect, giving it a dual role dependant on the hosts' conditions (Castro,P. 2006; Nikawa,H. 2006).

Development of the salivary pellicle onto enamel forms almost immediately after brushing, and consists of glycoproteins, acid rich proteins, mucins, exoproducts and sialic acid (Davies,T.M. 1991). Bacteria adhere to this layer through primary colonizers and surface interactions, followed by increasing colonization between cells (Davies,T.M. 1991; Marsh,P.D. 2005). Microbial counts increase through cell division forming a pellicle within 90 minutes of brushing (Quirynen,M. 1995). As colonization and multiplication of bacteria progress, the pellicle changes not only its conditions but also its inhabitants (Ahn,S.J. 2007).

Saliva has many beneficial properties. It serves as a buffer decreasing the solubility of hydroxyapatite, the major component in teeth. The buffering capacity helps prevent demineralization of teeth and neutralizes bacterial acid (Bardow,A. 2001; Bardow,A. 2000). In addition to buffering, salivary flow rate and total protein content affect bacterial counts and pH, thereby helping maintain equilibrium. In addition to quality, the quantity of saliva is important for buffering and caries resistance. Differences in gender and age as far as buffering capacity and salivary flow can affect caries prevalence. Increases in salivary flow not only increases pH, denying bacteria their optimum growth conditions, but copious saliva allows elimination of food and bacteria by way of swallowing. This form of

clearance, stimulated by chewing, can increase the amount of saliva and composition of saliva, furthering its importance (Lara-Carrillo,E. 2010).

Various components of saliva aid or deter bacterial attachment, directly affecting colonization, initiation and formation of caries. Antibacterial components such as lysozyme, lactoferrin, lactoperoxidase and secretory IgA prevent attachment of bacteria (Ahn,S.J. 2002; Castro,P. 2006; Gibbons,R.J. 1970; Quirynen,M. 1995; Radford,D.R. 1998). Proteins such as proline rich proteins, histatins and statherin can also inhibit bacterial adherence. Therefore, pellicles with decreasing levels of these particular proteins are found to have increased caries. This results from firmer attachment followed by bacterial growth (Ahn,S.J. 2008; Castro,P. 2006). In addition, salivary agglutins allow organisms to be removed through deglutination, by preventing binding and bacterial aggregation (Ahn,S.J. 2008; Castro,P. 2006; Jenkinson,H.F. 1997; Loimaranta,V. 2005).

Although salivary proteins can serve as bacterial antagonists, they can also promote bacterial adhesion by way of over forty proteins. Proteins serve as receptors for bacterial ligands or as a nutrient source, as in the case of sucrose dependant binding (Castro,P. 2006). Due to the very nature of high molecular weight glycoproteins and mucins, bacteria will readily adhere to a “ripened” pellicle (Gibbons,R.J. 1973; Jenkinson,H.F. 1994). In addition to bacteria binding by salivary proteins, saliva itself facilitates diffusion of nutrients necessary for growth. Proteins such as albumin, glycoproteins, mucin and sialic acid can all function in early colonization (Jenkinson,H.F. 1994; Jenkinson,H.F. 1997).

Saliva can also affect bacterial adhesion by masking the overall surface energy of a given material and negating its surface chemistry (Papaioannou,W. 2007; Quirynen,M. 1995; Radford,D.R. 1998). With surface energies leveled between two materials, unless receptors for a given bacteria are within the salivary pellicle, bacterial adherence will decrease (Quirynen,M. 1995). Saliva's effect on bacterial adhesion can be species dependant, based on a given bacterium's binding pattern (Ahn,S.J. 2007). Salivary interactions with bacteria that change their adhesion can alter the bacteria's genetic expression and the resulting biofilm (Pecharki,D. 2005). Therefore the patients' bacterial composition, along with any factors that could potentially change salivary flow and bacterial concentration is of great importance.

2.4 Orthodontics influence

Malocclusion or dental irregularity is a common oral health problem (Glans,R. 2003). Orthodontics aids to improve alignment for better oral hygiene and periodontal health, but research shows that only with average oral hygiene, does alignment help. In patients with good or even poor oral hygiene, alignment has no further effect on gingivitis (Alexander,S.A, 1991). Although studies have indicated orthodontics improves dental awareness and oral hygiene skills through frequent visits, it has been found that the oral bacteria of orthodontic patients is much different from those of healthy individuals. The difference is due to increasing levels of bacterial plaque (Batoni,G. 2001; Choi, 2009; Davies,T.M. 1991).

Plaque accumulation due to poor oral hygiene and a cariogenic diet can be compounded by fixed orthodontic appliances, which offer more surface area and mechanical overhangs. The introduction of orthodontic appliances increase areas where food debris can collect and increase the number of bacterial niches (Alves,P.V. 2008). Although it has been found that orthodontic appliances do not necessarily cause increased destruction of teeth or periodontal tissues, research demonstrates that bacterial loads are higher in orthodontic treated patients because of this lack of optimal oral hygiene (Alves,P.V. 2008; Sari,E. 2007). These opportunistic bacteria are capable of not only causing caries, but periodontal and fungal infections as well (Gudkina,J. 2008). In the case of those patients who are at increased risk of caries or periodontal disease, adjuncts such as fluoride, chlorhexidine and triclosan have been suggested (Atack,N.E. 1996). Most permanent damage to the dentition during orthodontic treatment is thought to be due to bad oral hygiene, not orthodontics. White spot lesions, which would otherwise be obsolete to a patient without appliances, are seen in 2-96% of orthodontic patients (Zachrisson, B.U. 1971). Even though these lesions rarely end up in caries, their progression is rather quick and leaves an indelible mark on the tooth and the patient post-treatment (Ogaard,B. 2001; Ogaard,B. 2006). These areas have been found to be rather resistant to normal measures of remineralization and last many years after appliances have been removed (Gorelick,L. 1982). This condition is exacerbated by the fact that at 6 weeks into retention, plaque levels can remain high and may not go away (Boersma,J.G. 2005). Today most orthodontic lawsuits are from patients' disapproval of their

white spot staining, regardless of their role in developing them. Although it's been found that carious lesions are linear to plaque values, it falls on the shoulders of the orthodontist when a patient does not have proper oral hygiene (Zachrisson,B.U. 1971).

Orthodontics can even affect salivary flow, buffering, pH and occult blood levels due to the fact that appliances can change the overall oral environment. Saliva flow increases with orthodontics, which in turn increase pH through increased levels of bicarbonate. Even though an acidic pH can be found in an orthodontic patient due to poor oral hygiene, increased salivary flow from the appliance can offset this (Chang,H.S. 1999; Lara-Carrillo,E. 2010).

Early caries and demineralization are often seen in orthodontic patients with poor oral hygiene (Petti,S. 1997; Sari,E. 2007). Whether this is due to appliances, retentive nature increasing plaque levels or reducing clearance, it is evident that appliances can aggravate an already compromised situation (Boersma,J.G. 2005; Kitada,K. 2009;). A greater concentration of plaque, leads to an increase in bacterial number, which in turn leads to more acid causing decalcification (Balenseifen,J.W. 1970). These developing white spot lesions have been documented in patients receiving orthodontic care and two of the main bacteria responsible are *Streptococcus mutans* (SM) and *Lactobacillus acidophilus* (LA) (Gorelick,L. 1982; Teanpaisan,R. 2007).

2.5 The Bacteria Involved

*Streptococcus mutans (*SM) is considered one of the main organisms in plaque that contributes to the initiation of caries (Nyvad,B. 1990). Despite being ubiquitous in the oral cavity, SM prevalence often indicates caries susceptibility and poor oral hygiene. SM is a gram-positive cocci, which uses food not only to adhere to the tooth, but also to produce its detrimental acids. This allows the bacterium to decrease the pH, preventing competitive bacteria from colonizing and eventually leading to early caries (Gizani,S. 2009; Gudkina,J. 2008), Although SM has a symbiotic relationship with other caries-causing bacteria, it is one of the most studied caries-causing bacteria. During orthodontics SM levels increase, but studies have indicated that levels of SM during retention, match the lower bacterial levels found during pre-treatment (Rosenbloom,R.G. 1991). Most pertinent for this study was the indication that clasps and acrylic from removable partial dentures resulted in an increase in SM levels (Mihalow,D.M. 1988).

Lactobacillus acidophilus (LA) is a gram-positive rod that is associated with the progression of caries. Like *Streptococcus*, it is consistently found as normal flora, only reaching greater proportions in extremely acidic conditions (Socranksly,S.S. 1971). LA is primarily found in areas of high carbohydrate or retentive surfaces enabling its preferred anaerobic conditions (Botha,S.J. 1993). Although LA is a late colonizer and favors SM for attachment, this bacterium produces a much stronger acid eventually suppressing SM growth (Lara-Carrillo,E. 2010; Smiech-Slomkowska,G. 2007; Svec,P. 2009).

2.6 Retention's Role

Retention is necessary for the majority of orthodontic patients post-treatment. Usually this retention consists of a retainer and patients are dismissed and supervised several times over the following years. At this point there is a shift in responsibility, from dentist to patient, and unfortunately the amount of relapse is dependant on diligent wear (Sheridan,J.J. 1993). Relapse in orthodontics is most often due to forces from periodontal fibers, oral musculature and growth. Often the general dentist is the first person to observe any changes in the dentition post-orthodontics, and long term studies investigating relapse, have led clinicians to opt for retention indefinitely (Little,R.M. 1988; Little,R.M. 1990). The aim of retention is to keep the teeth in position, but the overall appliance design can vary depending on the demands of the orthodontist and patient. The appliance needs to be durable and comfortable, while still being easy to adapt and overall effective for the orthodontist (Cerny,R. 2001; Cerny,R. 2008). According to a review published by the Cochrane Collaboration, current retention studies have insufficient research data and are unreliable for basing clinical decisions (Littlewood,S.J. 2006). Due to this, most retention appliances have been chosen based on clinical judgment and patient quality and satisfaction.

Removable retainers were developed to address some of the negative issues that are experienced by fixed lingual retainers. Removable retainers allow for better oral hygiene by not hindering oral hygiene methods (Cerny,R. 2001; Ristic,M. 2008). Removing the appliance makes it easier for a patient and

decreases their susceptibility to dental disease. The negative aspect of this appliance is that it requires patient compliance. Unfortunately, it is an appliance whose retentive nature and material makeup may deteriorate in the event of poor hygiene. In a study conducted by Kitada *et al*, researchers demonstrated that opportunistic bacteria and fungi levels were increased in all orthodontic patients when compared to those not receiving orthodontic care. More specifically patients with removable retainers had more dental plaque than non-orthodontic treated controls (Kitada,K. 2009). This indicates that although improvement in oral health may be seen after fixed orthodontics, the retention period is still at risk for oral health issues. Additional research done on removable appliances, found that bacterial levels in retainer patients were similar to partial denture wearers (Addy,M. 1982). But conflicting results do exist, implying that bacterial composition of retention patients may not differ substantially. For example; in comparison to non-appliance wearers, retainer patients have less bacteria in the buccal segment of the mouth, presumably due to dislodging bacteria with appliance removal (Arendorf,T. 1985). Although bacterial levels may increase with orthodontics, patients with removable retainers are similar to healthy non-appliance wearers demonstrating no increased gingivitis or periodontitis. In one study, the only microorganism that showed exceptional differences was that of the yeast, *Candida albicans* due to palatal coverage by PMMA (Petti,S. 1997). Due to the enormous differences in results between multiple studies, it is unclear the exact ramifications of retention appliances on oral health. Regardless of their

effect on oral health, the majority of removable retainers used today are the traditional Hawley retainer and the Vacuum formed retainer.

2.7 The Hawley Retainer

Developed in the 1920's the Hawley retainer, a tissue borne retainer, represents a large proportion of retainers currently used in orthodontics. It consists of acrylic, otherwise known as polymethyl methacrylate (PMMA), and has low solubility and toxicity (Theroux,K.L. 2003). Although generally referred for those patients where settling of teeth is needed, the retainer's positive attributes can be seen it its design (Figure 1). Acrylic palatal coverage with metal along the teeth, the design allows for subtle adjustments to the dentition. The Hawley retainer is known for its rigidity and long-term durability. These retainers are adaptable and often require more time, skill and money to fabricate and maintain (Sheridan,J.J 1993). Patient complaints range from embarrassment due to salivary flow and esthetics, to overall smell of appliance with age (Hichens,L. 2007). The materials of the Hawley retainer contribute to a specific set of problems. The acrylic lacks color stability and often shows shrinkage (Lewis,E.A. 1988). Methods to improve the mechanical properties, abrasion resistance, and solvent resistance are constantly being attempted to improve the quality of this retainer (Powers, 2006).

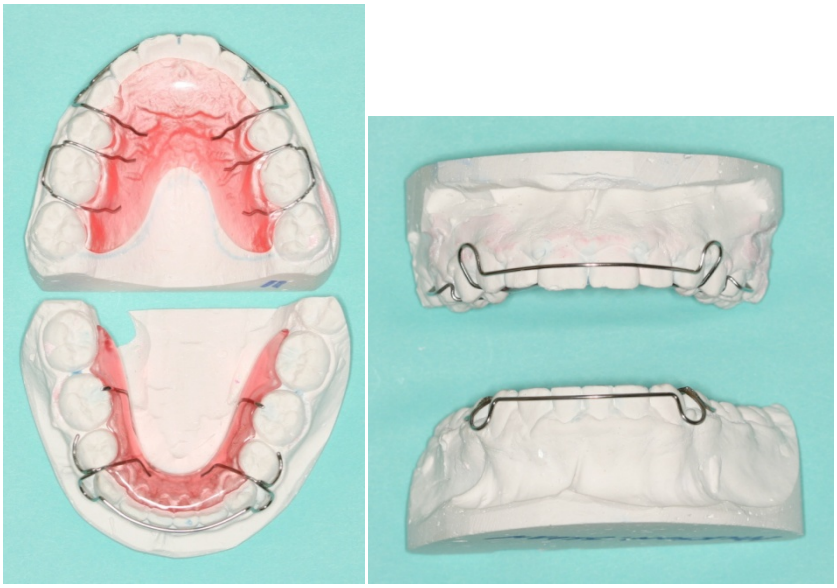

Figure 1: Traditional Hawley Retainer
The Hawley retainer from both the occlusal view and the view of the patient. It consists of both acrylic (PMMA) and wire clasps and bars for retention.

2.8 The Vacuum Formed Retainer

The vacuum formed retainer (VFR) is made of thermoformed polypropene or polypropene material (PP). Often referred to as an “Essix” by brand name, it is a clear, thin, full tooth coverage appliance that has become more popular in recent years with both orthodontist and patients (Figure 2). This popularity is due to its quick fabrication time, lowered cost and esthetic nature. Overall, the VFR is preferred over other removable retainers for its numerous benefits. The retainer limits palatal coverage preventing speech or hygiene issues often seen in the Hawley retainer, and is used to hold the dentition (Sheridan,J.J. 1993). This retainer requires less skill to produce and can vary in thickness to limit bulk (Lewis,E.A. 1988). In so far as retention, many studies have suggested that the VFR is no less effective than the Hawley retainer (Hitchens, L. 2007)

Unfortunately, its' new popularity in orthodontics, little research on its biological implications have yet to date be conducted (Lindauer,S.J. 1998).

Figure 2: The Vacuum Formed Retainer
The vacuum formed removable retainer is a thermoplastic polymer made from polypropene (PP). It is a thin clear plastic that after heating and suction, adheres to all surfaces of the tooth for retention.

2. 9 Biomaterial Implications

Due to the different biomaterials used for both the VFR and the Hawley retainer, differences in the oral microbiota may be present. When comparing the materials and each retainer's design, it can be presumed that they could cause very different oral health complications. The very nature of full tooth coverage by a VFR could serve as a reservoir for cariogenic bacteria, while the acrylic plate seen in the Hawley retainer could mimic the fungal environment found in similar denture base studies.

The Hawley retainer's bacterial concern deals mostly with the acrylic base plate that is tissue born. The acrylic base can be made from a variety of materials including, autopolymerized, heat-cured and triad visible light cured resins. Due to an extensive amount of studies on the bacterial adhesiveness of denture bases' and their relative fungal counts, many of the conclusions have been extended over to the Hawley retainer since PMMA is used for both appliances. This material is very absorbent to saliva and bacteria. In response, numerous attempts to improve its properties by cross-linking, adding nanofilled resins and modifying filler content or resin structure have been done (Hahnel,S. 2008). Unreacted monomer and lack of full polymerization can result in cracks or craze lines ultimately creating a safe haven for bacteria. Monomer and filler concentrations in addition to causing chemical irritation when leaching out, also attract plaque formation. More importantly is the retainer's unique position, allowing it to rest on tissue. This is compounded by its thickness and availability for bacterial binding (Lewis,E.A. 1988). Even the retainer itself, can prevent bacteria on the intaglio surface from being interrupted. It isolates bacteria from the oral musculature and saliva, allowing the bacteria to grow under their preferred conditions, acidic and anaerobic (Pusateri,C.R. 2009). Since a retainer can be prescribed for up to 24 hours of continuous wear, when caries are known to be more prevalent, it is exceedingly important that bacterial adherence of the retainer material is studied. In the case of SM, different amounts of monomers in PMMA have affected SM adhesion (Hahnel, 2008). The SM bacterial adhesion occurs despite the materials inherently low bacterial adhesion properties. SM

are attracted to high surface energy materials that are hydrophilic, while PMMA is rather hydrophobic. However the Hawley retainer does consist of metal portions, which could explain a higher attachment created from a slightly increased surface energy. Studies show chemical adjustments within the PMMA, such as double cross linking, produce the lowest streptococcal attachment (Hahnel,S. 2008). On the other hand, PMMA that is coated with chemicals, may somehow serve as a receptor for bacterial binding (Radford,D.R. 1998).

The only test on bacterial adherence of VFR material compared to PMMA was done by Lewis *et al* in 1988. Here the authors tested different types of PMMA, such as autopolymerized, heat cured and visible light cured against the thermoplastic “Biocyrl”. Scanning electron microscopy was used to compare surface characteristics, which are believed to affect bacterial adherence. In the study Biocryl resin was somewhat smoother in surface roughness, possibly leading to its decreased bacterial adherence. This smoother Biocryl resin had less adhered gram-positive and negative rods, which supports previous studies suggesting surface roughness leads to better bacterial adhesion. Lewis also showed that heat cured PMMA, often the kind used for making a Hawley retainer showed the most bacterial adherence, specifically by SM. Importantly, SM adhered more or to the same extent to acrylic, as it did to enamel. Regardless of increases in bacterial adhesion, this study demonstrated that the subgingival flora did not change, implying that the periodontal condition of the patient was not affected (Lewis,E.A. 1988). Unfortunately, VFR material or PP has not been investigated for bacterial adhesion properties since this initial study.

Of utmost concern to patients wearing a VFR is the increased possibility for demineralization of tooth structure (Sheridan, J. 2001). In a case study presented by Dr. Birdsall, a patient was described to have severe caries and demineralization of tooth structure with full time wear of a VFR retainer during a diet high in cariogenic drinks. This patient had extreme sensitivity to hot and cold and therefore was unable to eat or drink without the retainer. The patient was also unable to brush due to his sensitivity. It was concluded from the patient's pattern of carious habits and demineralization, that the constant wearing of this tooth covered retainer while consuming soft drinks, allowed the acidic carbonation to pool around teeth, preventing the protective and buffering ability of saliva to neutralize the low pH in this area (Birdsall,J. 2008). The possibility of demineralization becomes especially important for teenagers because they have higher than average cariogenic diets and serve as the predominant patients in an orthodontic practice. In a study by Ogaard *et, al.,* researchers found that demineralization can be seen as early as one month after orthodontic appliance placement (Ogaard,B. 1989; Ogaard,B. 2006). Therefore demineralization is a consideration when deciding what retainer to be chosen for a particular patient. In instances of patients with poor oral hygiene, compliance issues or a high caries risk, it may be advisable to avoid VFR usage (Birdsall,J. 2008).

Bacterial adherence is needed for growth and differs between various materials (Bollen,C.M. 1997; van Houte,J. 1994). Adherence allows continued shelter from the biological processes normally used for their removal. Often bacterial growth flourishes, making the bacteria more resistant over time. The

bacteria can now communicate by increasing receptors or secreting components which facilitates additional binding between different bacterial species (Appelbaum,B. 1979; Doyle,R.J. 1995; Gibbons,R.J. 1973). *Streptococcus* specifically, has been found to increase adhesion on prosthesis when in the presence of *Candida* (Pereira-Cenci,T. 2008). For this reason dental materials are manufactured in hopes of a low susceptibility to plaque bacteria, otherwise an additional treatment protocol of fluoride may need to be considered (Nikawa,H. 2006; Pereira-Cenci,T. 2008). Initially, bacterial adhesion is due to the elemental and molecular makeup of the material, which affects its affinity for bacteria through hydrophobicity, hydrogen bond capacity and electron potential. Therefore, surface free energy plays a large role in bacterial adhesion. Bacteria usually have high surface energy, while saliva's surface free energy remains low. In addition, bacteria tend to bind materials with surface free energy similar to their own. Therefore, materials with high surface energy attract more plaque. On the other hand, lower surface energies decrease adhesion initially, decreasing a bacterium's overall binding force (Pereira-Cenci,T. 2007).

More importantly, the roughness of a material overrules the surface energy difference between two materials. Roughness is specific to the material, and depends on the material's inherent properties as well as the impact of modifications made during fabrication by dental technicians (Busscher,H.J. 1984; Papaioannou,W. 2007). In the case of PMMA, differences in material properties can vary depending on the amounts of certain chemicals and consistency of mixing (Gedik,H. 2009). Cytotoxicity of monomer has been known to have an

antibacterial effect, while fillers often used to increase wear resistance can make a material rough (Ahn,S.J. 2006; Bollen,C.M. 1997). Although there is no increased attraction for bacteria to rough materials, it is the voids, which either protect bacteria or allow additional time for growth, thereby increasing their number (Nyvad,B. 1993). These irregularities allow bacteria to be stagnant and thicken. This increased species diversity changes to a rather irreversible binding (Thomas,R.Z. 2008). Studies have measured the effect of roughness, finding two to four times the amount of adhesion with rough materials (Quirynen,M. 1994; Quirynen,M. 1995). Roughness is measured by the spaces between irregularities. It allows the initial adhesion seen in cracks, grooves or abrasion, and is thought to be species specific (Bollen,C.M. 1997; Quirynen,M. 1994; Quirynen,M. 1995). SM specifically, although found on smooth surfaces, has increased adherence on rough or porous material (Pusateri,C.R. 2009).

Fabrication adjustments such as polishing, changes the overall properties of a material by altering its relative roughness (Thomas,R.Z. 2008). Methods tested indicate that differences in procedures can increase surface roughness up to ten fold. But for the most part, the laboratory technique of polishing can decrease the overall roughness of a material if done using routine protocols (Bollen,C.M. 1997). Because roughness was found to speed up colonization, studies were done to standardize treatments for different materials. Through the use of various polishing techniques, it was discovered that there is a threshold for roughness that determines if a surface is plaque retentive or not. As long as the materials roughness is less than 0.2µm, bacterial attachment differences are

insignificant with change in roughness (Bollen,C.M. 1997; Quirynen,M. 1995). Most studies were done on PMMA and it can be noted that even with standardized protocols for laboratory fabrication, often a retainer would undergo damage from brushing or common cleansers (Bollen,C.M. 1997; Samaranayake,L.P. 1980). Wear overtime could result in areas where roughness is above threshold roughness and bacterial attachment is increased. Another factor affecting this adherence is the larger size and shape of the retainer, compared to the VFR. Often the larger surface area available for colonization, the more bacteria are adhered (Papaioannou,W. 2007). Although the VFR material is much smoother overall than the PMMA, it should be mentioned that the edges of this retainer could possibly serve as bacterial attachment sites. Being that there is no research on the bacterial adherence of this newer VFR material or formal protocols for polishing, it is very possible that this material, when left rough or cracked, may harbor additional plaque bacteria.

The purpose of this study is based on the fact that two different materials may have two entirely different relationships with bacteria, based on their design, position and physiochemical makeup. A retainer placed in an orthodontic patient, allows plaque to become stagnant. This sheltered environment can change the microenvironment and possibly lead to disease. Knowing a material's susceptibility to caries-causing bacteria, allows clinicians to consider another element when selecting the appropriate retainer for patients, especially those that are caries prone.

CHAPTER 3

METHODOLOGY

3.1 Experimental Design

An *in vitro* randomized study was done on two commonly used orthodontic retention materials, self-cure polymethyl methacrylate (PMMA) and the Thermoform Polymer (Visacryl C) made of polypropene (PP), to test their relative adherence by two common caries causing bacteria, *Streptococcus mutans* (SM) and *Lactobacillus acidophilus* (LA). Discs were fabricated from both materials and either subjected to saliva, bacteria or both for extended amounts of time in polystyrene tissue culture plates. In order to determine each material's relative adherence for each bacterial species, cultural analysis and metabolic assays were done. All studies were conducted in triplicate after initial methods testing was completed. Controls were included to prove aseptic technique and lack of contamination throughout the study. The experimental design included both positive and negative controls. Negative controls contained no microorganisms or saliva. Positive controls consisted of saliva only and bacterial carry over resulting from the liquid's attraction to the disc itself.

3. 2 Bacterial Culturing and Cell Concentration

Two isolates of oral bacteria were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and were cultured according to ATCC instructions. *Streptococcus mutans* (SM) ATCC 25175 (NCTC 10449), isolated from carious dentine, was thawed, streaked and cultured on blood agar plates

consisting of Trypticase soy agar with 5% defibrinated sheep's blood (Difco, Sparks,MD). *Lactobacillus acidophilus (LA)* ATCC 3456 was cultured on Man Rogosa Sharpe agar (MRS; Difco). After growth on their respective agar plates, the bacteria were inoculated into Trypticase soy Broth (TSB; Difco) for SM and MRS broth for LA. From the initial overnight liquid cultures, glycerol stocks for long term frozen storage were made. Liquid bacterial cultures were prepared by innoculating 1ml of the appropriate broth with single colonies isolated from the agar plates. The 1 ml cultures were grown overnight aerobically at 37°C The saturated 1 ml cultures were used to inoculate 25 ml of pre-warmed broth, and incubated at 37℃ with rotary shaking of 90 RPM. The overnight 25 ml liquid culture was used to inoculate 125 ml of broth and the bacterial growth was monitored by measuring the turbidity of the samples with a spectrophotometer at an optical density of 650 nm. Bacterial cell concentrations were determined by creating a standard curve of measured absorbance versus measured enumeration of CFU. Prior to harvesting, the bacteria cultures were diluted to allow cells to re-enter exponential growth phase. Starting with an absorbance of 0.5, the optical density of the cells was monitored every 30 minutes until the cells reached an absorbance of 0.8 OD. Cells were harvested by centrifugation at 7700xg for 5 minutes at 37°C and then resuspended with fresh media.

To determine bacterial cell number, both SM and LA liquid cultures were grown to an OD of 0.8 at 650 nm. The washed cells were then serially diluted and plated onto their respective agar plates to calculate the number of cells representative of that absorbance. *Streptococcus mutans* and *Lactobacillus*

acidophilus resulted in a $1X10^8$ CFU/ml at an absorbance of 0.8. For all subsequent experiments, bacteria were grown to an absorbance of 0.8 and the cells harvested by centrifugation and resuspended in fresh broth. The bacteria were then added to experimental wells of a 48-well Costar plate at a final concentration of $3X10^8$ CFU per well and incubated at 37℃ (Pereira-Cenci,T. 2008).

3.3 Materials Tested

Discs were fabricated from two commonly used retainer materials, PMMA and PP as described below. Both types of discs were prepared to identical size and dimensions. Discs were disinfected with 80% ethanol, rinsed several times and stored in sterile water to leach out excess monomer. After thorough sterilization and rinsing, discs were air dried and stored in a sterile Petri dish (Serrano-Granger,C. 2005). No surface modification was performed after the discs were processed (Pusateri,C.R. 2009). Defective discs showing any obvious surfaces imperfections were discarded (Serrano-Granger,C. 2005).

3.4 Fabrication of PMMA Discs

Heat-cured polymethyl methacrylate (PMMA), (Great Lakes Lab, Tonawanda, New York) disks (6 mm X 1.5 mm) were fabricated by Great Impressions Lab (Las Vegas, NV) according to manufacturer's instructions (Serrano-Granger, C. 2005). Metal washers with an internal circumference of ¼ inch, matching that of the standard paper punch were used as molds to fabricate the disks. PMMA was

mixed according to manufacturer's recommendations and fabricated at Great Impressions dental laboratory to match clinical specifications. PMMA was prepared using 1 part self-polymerizing acrylic powder and 0.8 part monomer liquid. The mixed polymer was immediately placed inside the washers, with one side facing type 3 dental stone. After curing with a pressurized cooker, discs were separated from their molds (Pereira-Cenci,T. 2007). No polish or finish was performed, but only discs with relatively smooth edges were used for experiments. Discs were then disinfected with 80% ethanol for one minute and placed in distilled water for one week to leach out any excess monomer (Tanner,J. 2003). Discs were later dried and kept in sterile Petri dishes until used in experiments.

3.5 Fabrication of Thermoform Polymer Discs

VFR material, a thermoplastic polymer of PP, was formed into discs using Invisacryl C provided by Great Lakes laboratory. This material was chosen at a thickness of 1.5 mm, to match that of washers similar to those used to fabricate acrylic discs. After the blue protective coating was removed, a standard hole punch with a diameter of ¼ inch was used to create discs of equal size and proportion. No surface modification was done to match that of a clinical situation. The round polymer disc, was then sterilized with 80% ethanol, rinsed and stored in sterile distilled water (Tanner,J. 2003). Prior to the experiment, discs were set to dry in a sterile Petri dish.

3.6 Collection and Preparation of Early salivary pellicle

Unstimulated whole saliva was provided by the investigator by expectorating into a chilled sterile 50 ml polypropene tube (BD Bioscience, San Jose, CA). Collected saliva was pooled and different methods for clarification and sterilization were investigated. The saliva samples were then incubated at 37℃ and monitored for bacteria and fungal growth to determine the optimal method for preparing a sterile salivary pellicle (Muller,R. 2009).

3.7 Salivary Pellicle Pre-conditioning

Saliva (approximately 15 ml) for the experiments was processed according to optimal methods determined above. The saliva was homogenized by vortexing for one minute to reduce viscosity of the sample and subsequently clarified by centrifugation at 4000xg for 20 minutes at 25°C to remove any cellular debris or food particles. The precipitate was discarded, and the resulting supernatant was filter-sterilized using a 0.2µm cellulose acetate membrane (Millipore Billerica, MA), (Papaioannou,W. 2007). Saliva was stored at 4°C for immediate use according to Hahnel (Hahnel,S. 2008). Experimental discs were placed into Costar 48 well plates (Corning, Lowell, MA) and incubated with 200µl of conditioned saliva for one hour at 37°C. Discs were then removed from saliva and immediately placed into 24 well plates and the appropriate bacterial suspension was added (Hahnel,S. 2008; Kitada,K. 2009).

3.8 Biofilm/Adhesion Assay

All studies were performed in triplicate on sterilized discs of equal size. The adherence of both SM and LA to both test materials, PMMA and Thermoplastic Polymer were examined. $3X10^8$ CFU of bacteria was added to wells containing the discs. The plate was incubated at 37℃ for 2 hours o f adhesion. The bacteria were left undisturbed until the discs were rinsed three times with Phosphate butter saline (PBS) to remove the non-adherent cells (Hahnel,S. 2008). Wells containing un-inoculated media incubated with the test materials were used as negative controls. Positive Additional experiments were conducted to determine if discs not coated in saliva had different adherence properties and to determine the amount of non-adhered bacteria that may inadvertently be counted as adhered bacteria reflecting carry over. For the carry over experiment, the disc, after salivary pellicle formation, was quickly dipped into $3X10^8$ CFU of bacteria and then immediately rinsed three times with PBS. This served as a control for any bacterial carry over due to hydrophilic attraction of the bacteria to liquid on the disc. Both positive and negative controls served to verify these methods and to assess sterility and aseptic technique.

3.9 Effect of Early Salivary Pellicle

In order to examine the effect of saliva on bacterial adhesion, acrylic discs were conditioned with and without 200 µl of saliva in a 48-well plate. Discs were incubated with saliva for one hour prior to incubation with the bacteria.

(Papaioannou,W. 2007). After incubation with or without salivary pellicle, discs were transferred to new wells containing 1 ml of $3X10^8$ CFU of bacteria for a two hour incubation at 37°C. Discs were then washed by dipping into fresh PBS three times to remove non-adherent cells, prior to any quantification.

3.10 Effect of Material on Adhesion

Sterilized PMMA and PP discs were pre-treated with 200 µl of saliva in a 48-well plate for one hour. The discs were then placed directly into wells of a 24-well tissue culture dish and inoculated with at total of $3X10^8$ cells in 1 ml of broth. The plates were incubated aerobically at 37°C as a static culture, without shaking. After two hours of incubation, discs were removed from the bacteria containing wells and dipped into fresh PBS three sequential times to remove non-adherent cells prior to quantification of the number of adherent bacteria.

3.11 Determination of Number of Adhered Bacteria : CFU Counting

After rinsing in PBS, the discs were swabbed with sterile cotton swabs. Swabs were placed into centrifuge tubes containing 400 µl of PBS. The swabs were twirled in the PBS to release the bacteria and the wooden stick was cut off and tubes were centrifuged to pellet the bacteria prior to discarding the swab. The bacteria were then resuspended in ten-fold serial dilutions with PBS and plated to analyze cultures. All discs were analyzed in triplicate followed by ten-fold dilutions in order to calculate CFU for comparisons. Then 50ul aliquots of the dilutions were plated onto appropriate agar plates and incubated aerobically at

37°C for 48 hours. After incubation, colony forming units (CFU) were enumerated as the unit of adhesion and then photographed with a camera for verification (Papaioannou,W. 2007).

3.12 Assessment of Cellular Metabolic Activity in Adhered Cells

Adherent cell viability was tested using the colorimetric reduction assay tetrazolium sodium 3'-{1-[(phenlyamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)-benzene sulphonic acid hydrate (XTT, Sigma, St Louis, MO). The assay quantifies metabolic activity and was used in this study to determine the relative number of adherent cells. For this assay, discs previously incubated with bacterial cultures and washed three times with PBS, were placed into fresh wells containing media for SM or PBS for LA (Islam,B. 2008; Pereira-Cenci,T. 2007). XTT solution was prepared by dissolving 0.5 mg/ml of tetrazolium salt, XTT (Sigma) and 40 µg/ml CoenzymeQ0 (Sigma) (Taweechaisupapong,S. 2010). A 60 µl aliquot of pre-made XTT solution was then added to all wells containing 240µl of media for SM or PBS for LA. Discs containing adhered SM were assayed using 240 µl of fresh media, while discs containing adherent LA required PBS due to the medium's dark color. The XTT reagent was added to each well according to manufacturer's recommendations, and plates were incubated in the dark aerobically at 37°C (Islam,B. 2008). Control wells contained media only, discs not pre-treated with saliva and a carry over disc, which was dipped only in bacteria suspension without incubation. After four hours, 100 µl of solution from each well was removed and the absorbance read

in a 96-well plate (Choi,D.S. 2009). An orange colorimetric change directly correlates with the metabolic activity of the biofilm and hence gives an indication of the number of bacteria present. The colorimetric change was measured with a plate reader at 492 nm (Bio-tek Instruments, Inc., Vermont) (Taweechaisupapong,S. 2010).

3.13 Performance of Statistical Analysis

For both adherence-testing methods, statistical analyses compared the adherence of SM and LA on PMMA and PP. Adherence testing assessed by enumerating adhered CFU was compared for statistical significance with a Students T-Test. Adherence testing by XTT proliferation assays used a T distribution test to analyze the mean of a normally distributed sample due to the experiments' small sample size. Data were considered statistically significant if the P value was < 0.05.

CHAPTER 4

RESULTS

The goal of this research was to determine if any difference existed in the adhesion of SM and LA onto the two retainer materials PMMA and PP. Discs were fabricated according to standard clinical parameters for fabrication of both retainers. Discs were treated with or without saliva, prior to bacterial adhesion by SM and LA to determine if the presence or absence of saliva altered the inherent bacterial adhesion properties of the materials. After bacterial adhesion, the number of adhered bacteria on both materials was quantified by counting the number of recovered CFU. Relative quantitation of the number of adhered bacteria was also assessed using a colorimetric assay of bacterial metabolic activity (XTT assay).

4.1 Salivary method testing

Initial experiments were performed to determine the most appropriate conditions for the collection and processing of saliva for experimental use. Whole saliva (WS) was clarified using centrifugation at either 4,000 x g or 7,500 x g for 20 minutes at 25℃ to remove cellular debris. The clarified fractions were then incubated with sterile TSB (Tryptic Soy Broth) or MSB (Minimal Salts Broth) and monitored at 12 and 24 hours to determine if centrifugation alone was sufficient to remove salivary contaminants. The results depicted in Figure 3 clearly demonstrate that all centrifuged saliva samples facilitated bacterial and/or fungal growth that was not present in sterile TSB or MSB wells. Whole saliva (WS) was

compared to TSB and MSB media only for detection of contamination. Cloudiness of media was seen as contamination (+) when compared to that of clear uncontaminated (-) media. Results indicate centrifugation alone was not sufficient to clarify the saliva and remove these contaminants. Based upon this evidence, whole saliva (WS) was once again clarified using centrifugation at either 4,000 x g or 7,500 x g for 20 minutes at 25℃ to remove cellular debris. The clarified fractions were then filtered using a 0.2 µm filter and stored at 4℃ for analysis. The clarified, filtered fractions were incubated with sterile TSB or MSB for 24 hours to determine if the combined processes were sufficient to remove bacterial contaminants (Figure 4). These results clearly demonstrated that all centrifuged and filtered saliva samples (0.2 µM) were sufficient to clarify the saliva and remove these contaminants. On the other hand, unfiltered samples resulted in bacterial growth and contamination, shown in Figure 4 as positive (+). No bacterial or fungal growth was present in the control samples of sterile TSB(n=24) or MSB(n=24) wells, shown below as negative (-) or lack of cloudiness (Figure 4).

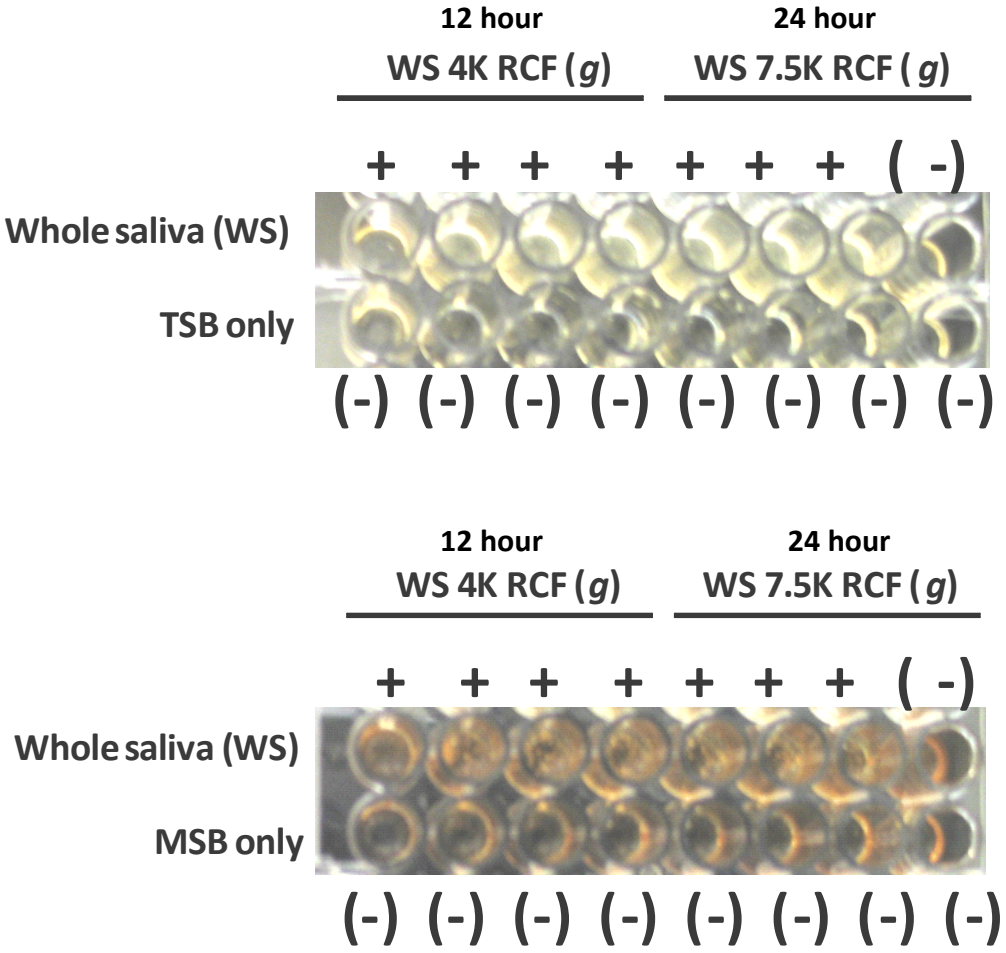

Figure 3: Saliva methods testing

Saliva was unfiltered and centrifuged at 4,000xg and 7,500xg before incubating at 37℃ for 12 and 24 hours. Whole saliva (WS) was com pared to TSB and MSB media only for detection of contamination. Cloudiness of media was seen as contamination (+) when compared to that of clear uncontaminated (-) media. Results indicate centrifugation alone was not sufficient to clarify the saliva and remove these contaminants.

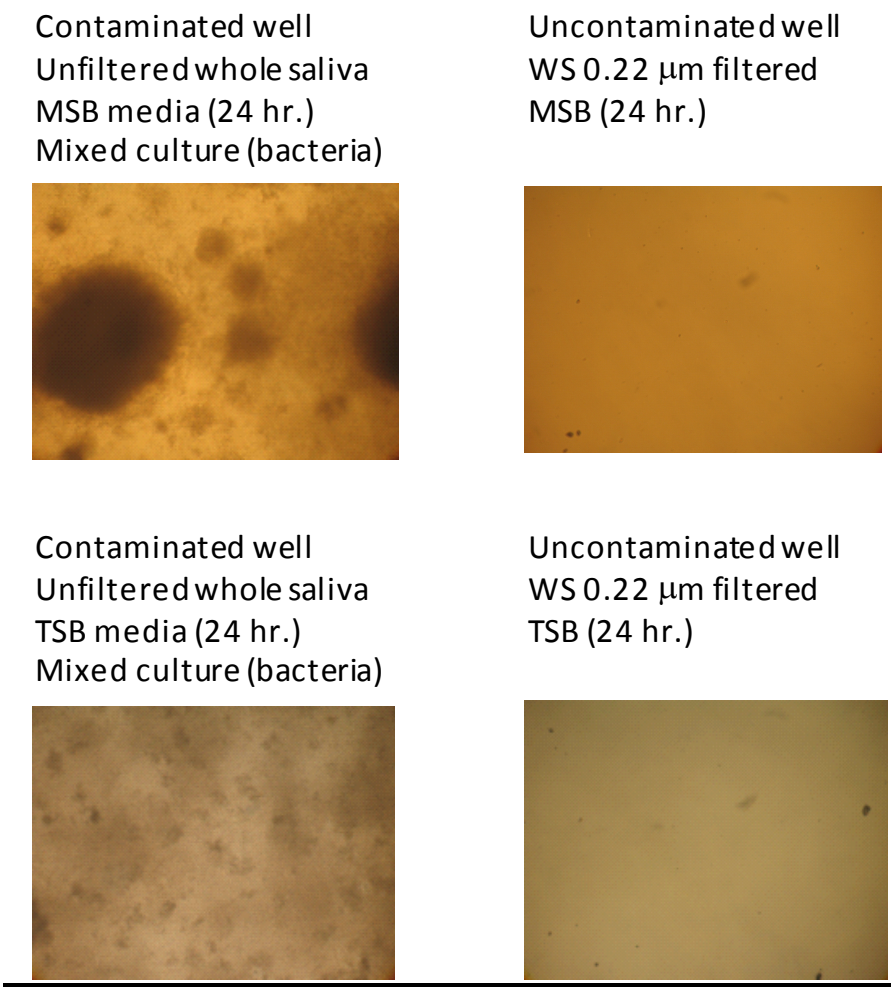

Figure 4: Saliva sterility
Images of saliva filtered and unfiltered, in MSB and TSB, after incubation of 12 and 24 hours. Unfiltered samples resulted in contamination, seen as bacterial growth. Filtered samples show no bacterial growth. These results clearly demonstrate filtration is necessary to sufficiently clarify saliva samples and remove bacterial contaminants.

4.2 CFU Adhesion Method Testing

SM and LA were incubated with saliva for one hour followed by aerobic incubation for bacterial adherence for two hours. Discs were then rinsed three times with PBS, swabbed and ten-fold dilutions plated for calculating the CFU of attachment. CFU were enumerated at 48 hours. Figure 5 shows verification of saliva sterility for adhesion plating. The saliva used for pre-conditioning was plated and incubated aerobically for 48 hours at 37℃. The absence of colonies on the plate confirms that the saliva isolation and preparation methods previously tested; do in fact produce sterile saliva.

Figure 5: Adhesion Plating Saliva Sterility Controls
Sterility of saliva was checked on each bacteria's respective plate. The saliva used for pre-conditioning was plated and incubated aerobically for 48 hours at 37℃. Plate shows that previously tested methods, using filtered saliva, do in fact produce sterile saliva.

Preliminary experiments were also conducted to determine the experimental conditions which minimized carry over of non-adherent bacteria caused by hydrophilic interactions of rinse liquid's attraction to the disc. Both PMMA and PP discs were pre-conditioned with saliva and quickly dipped into 1

ml of both bacterial cultures for one second prior to testing different methods of washing to decrease carry over of non-adherent bacteria. The different methods tested included suction pipetting of surrounding media, removing the disc from the well and placing into two successive wells containing PBS, and vigorous washing of the discs in three successive wells containing PBS. The results demonstrated that carry over of non-adherent bacteria due to surface interactions does exist (Figure 6). Presumably, these liquid droplets are attracted to the disc and can contribute background CFU to the experiments. An analysis of the results determined that three successive washes in PBS resulted in the least amount of non-adherent bacterial carry over. During subsequent experiments, attempts were made to further decrease the amount of residual liquid and non-adherent bacteria remaining on the disc by touching the disc to the side of the sterile well prior to swabbing the discs.

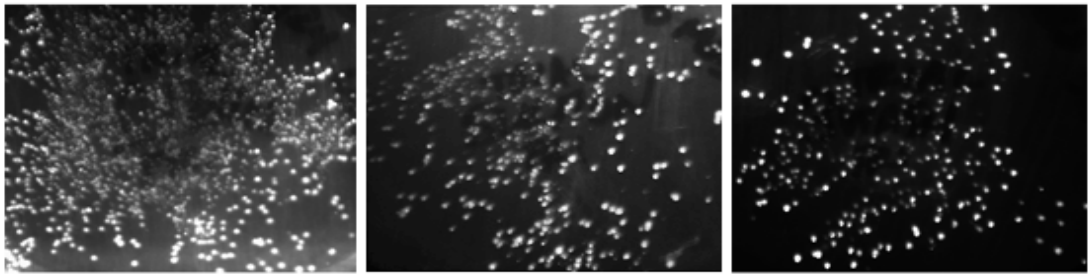

Figure 6: Methods Testing to Decrease Carry Over
Methods were tested for decreasing carry over due to hydrophilic interactions of liquid's attraction to the disc itself. Discs were pre-conditioned with saliva and dipped into bacterial cultures for one second before testing methods for decreasing carry over. Methods tested were suction pipetting of surrounding media, washing two times with PBS or vigorous wash three times with PBS. Determination was made that three washes in PBS decreased carry over the most.

Although all methods tested resulted in some non-adherent bacteria carry over or background, a comparison of the number of CFU produced by the background carry over and that from actual adherent bacteria demonstrated the amount of bacterial carry over is insignificant. Figure 7 shows the comparison of bacterial CFU for both the carry over well and for the number of CFU present after a 2 hour adhesion incubation. For the carry over well trials, both PMMA and PP discs were pre-conditioned with saliva and quickly dipped into bacterial cultures of $3X10^8$ cells/ml. Carry over discs were then washed in PBS three times and swabbed. Swabbed samples were then resuspended in 400µl of PBS and plated for 48 hours at 37℃. Comparisons were then ma de between a disc that is incubated with bacteria for 2 hours, from the carry over disc which was momentarily dipped into culture prior to plating. Figure 7, panel A illustrates the number of CFU obtained from swabbed discs after 2 hour adhesion incubation. Panel B illustrates the number of CFU obtained from the carry over experiment. These results clearly demonstrated that the amount of carry over bacteria is several orders of magnitude less than the number of adherent bacteria and can be considered insignificant to contributing to the analysis of adherent bacteria.

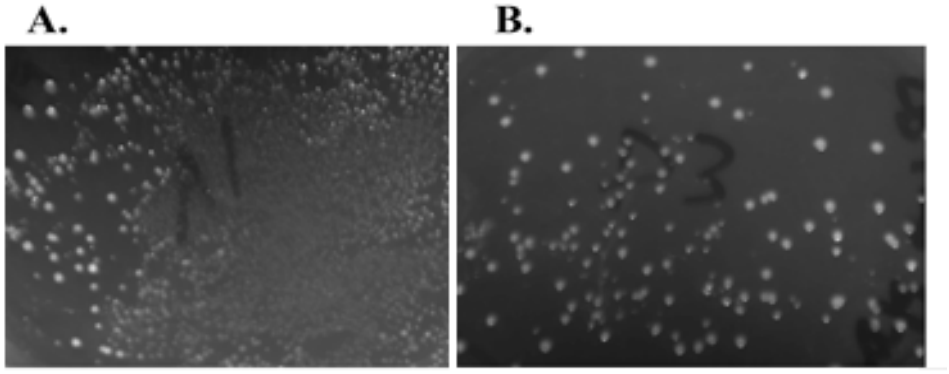

Figure 7: Adhesion Plating for Carry Over Controls
Bacterial carry over created by hydrophilic interactions (B) is shown above in comparison to plates of bacterial incubation for adherence of 2 hours (A). Discs were pre-conditioned with saliva were dipped momentarily in bacterial cultures, followed by three immediate washes in PBS. These discs were then swabbed and samples plated and incubated for 48 hours at 37℃. Above plates (B) show carry over does exist due to bacteria within liquid droplets. These droplets are attracted to the disc at time of swabbing and serve as background. When carry over plates are compared to the concentration of a bacterial adherent plate (A), it is clear that hydrophilic attraction on the carry over plate is much lower than those bacteria actually adherent. Since carry over (B) is much lower than that of an incubated plate (A), bacterial carry over was considered background.

4.3 Results of Culture Analysis

Discs of both materials were pre-treated with sterile saliva prior to incubation with both bacteria. Discs were then rinsed in PBS, swabbed and ten-fold dilutions were done for plating on their respective agar. Diluted bacterial swab samples were plated for 48 hours prior to CFU counting. Table 1 shows the results from these studies. All plates containing more than 300 colonies were considered too numerous to count (TNTC). Although CFU values differed between triplicates, increased adhesion for PMMA in comparison to PP was seen with both bacterial

species, when comparing plates of corresponding dilutions and groups. CFU averages were calculated from triplicate plates that contained CFU between 30 and 300, allowing direct numerical comparison between the different materials. This range of 30 to 300 CFU is the standard protocol for accuracy in counting colonies. Table 2 shows this average indicating a three fold increase in adhesion of both bacteria to PMMA when compared to PP.

CFU/ml Adherence for *Streptococcus mutans* (SM)
and *Lactobacillus acidophilus* (LB)
On Polymethyl methacrylate (PMMA) and (PP)

Plate Dilutions In triplicate	SM PMMA	SM PP	LB PMMA	LB PP
10^{-2}	TNTC TNTC TNTC	199 113 70	TNTC TNTC TNTC	TNTC TNTC TNTC
10^{-3}	46 13 47	10 2 2	95 TNTC TNTC	32 186 TNTC
10^{-4}	4 2 2	0 0 0	13 30 50	3 20 8
10^{-5}	0 0 0	0 0 0	1 4 6	2 4 3

Table 1: CFU/ml for Bacterial Adherence
CFU were counted for both SM and LA on both PMMA and PP in ten-fold dilutions. Overall both bacteria were more adherent to the PMMA than PP when comparisons between plates of specific dilutions were done. Values are CFU counts for triplicate plates at each specific dilution. Colonies over 300 were considered TNTC.

Average CFU/ml Adherence for *Streptococcus mutans* (SM) and *Lactobacillus acidophilus* (LA) on Polymethyl methacrylate (PMMA) and Polypropene (PP)				
Bacterium Material tested	SM PMMA	SM PP	LA PMMA	LA PP
Average CFU/ml	$3.5X10^4$	$1.3X10^4$	$3.1X10^5$	$1.1X10^5$

Table 2: Average CFU/ml for Bacterial Adherence
CFU were counted for both SM and LA on both PMMA and PP. Values are averages of CFU counts for triplicate plates, that fall between the range too few (30 CFU) and too numerous to count (300 CFU). Overall both bacteria were more adherent to the PMMA than PP when comparisons between plates of specific dilutions were done.

Figures 8 and 9 illustrate the plate comparisons between PMMA and PP for each target organism. Images were taken to document those plates where colonies were TNTC, preventing direct numerical and statistical comparison. Discs were preconditioned with saliva and plated with bacteria for the adhesion testing of both PMMA and PP for 48 hours at 37℃. The plates shown are ten-fold dilutions of the eluted bacteria from both PMMA and PP discs. A visual comparison of the plates indicates that adhesion to PMMA is greater than PP, for both SM and LA.

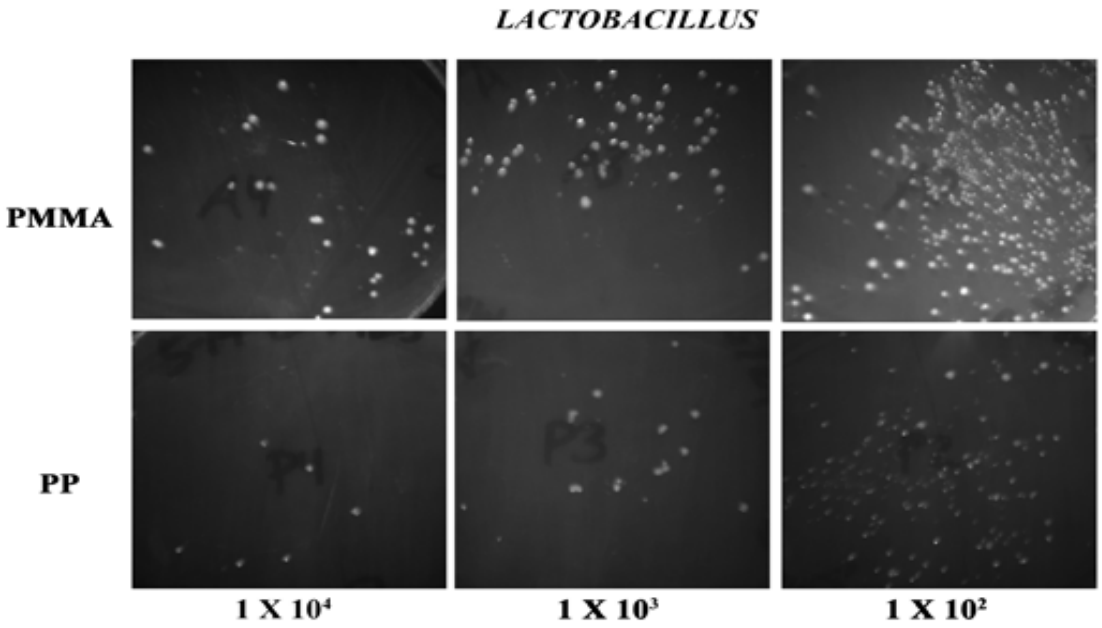

Figure 8: Comparison of LA Adhesion Plates for PMMA and PP
Discs were preconditioned with saliva and exposed to LA for adhesion testing of both PMMA and PP for plating and incubation of 48 hours at 37℃. Plates shown are ten-fold dilutions of eluted LA for both PMMA and PP. Direct visual comparison showed greater number of CFU for PMMA than that obtained for the PP.

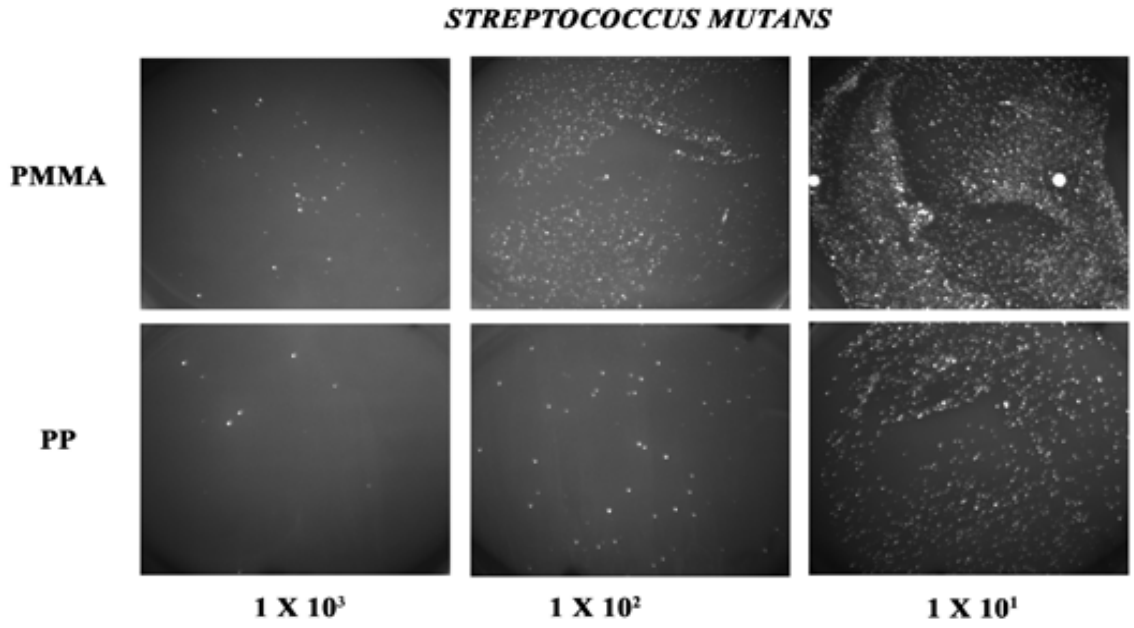

Figure 9: Comparison of SM Adhesion Plates for PMMA and PP
Discs were preconditioned with saliva and eluted SM was plated for adhesion on both PMMA and PP for 48 hours at 37℃. Plates shown a re ten-fold dilutions of SM for both PMMA and PP. Direct visual comparison showed greater numbers of CFU eluted from PMMA than PP.

The significance between CFU for both bacterial species and materials on plates that were able to be enumerated was further compared and the results illustrated in Figures 10 and 11. These dilutions (10^{-4} for LA and 10^{-3} for SM) serve as those plates containing a range of CFU enumerated for statistical and graphical comparison. CFU for triplicate plates were averaged for PMMA and PP, Figure 10 represents the adherence comparison of LA at 10^{-4} dilutions. Overall PMMA had a 3-fold increase in the amount of LA adherent compared to PP.

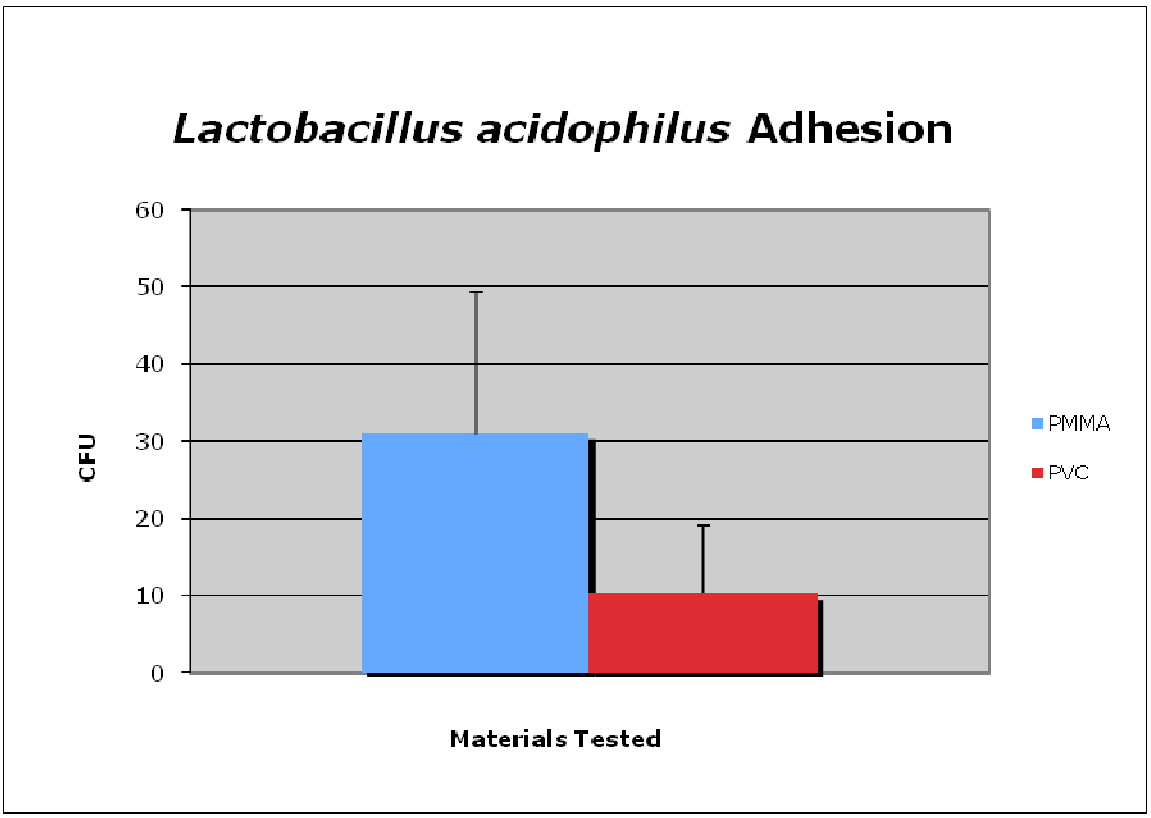

Figure 10: CFU of *Lactobacillus acidophilus* Adherent to PMMA and PP. Adherence was charted for LA at 10^{-4} dilutions for comparison of adhesion values. CFU's for triplicates were averaged for PMMA and PP. Overall PMMA had an increased amount of LA adherent compared to PP, calculating to roughly a 3-fold difference. Error bars represent the difference in bacterial counts between triplicates.

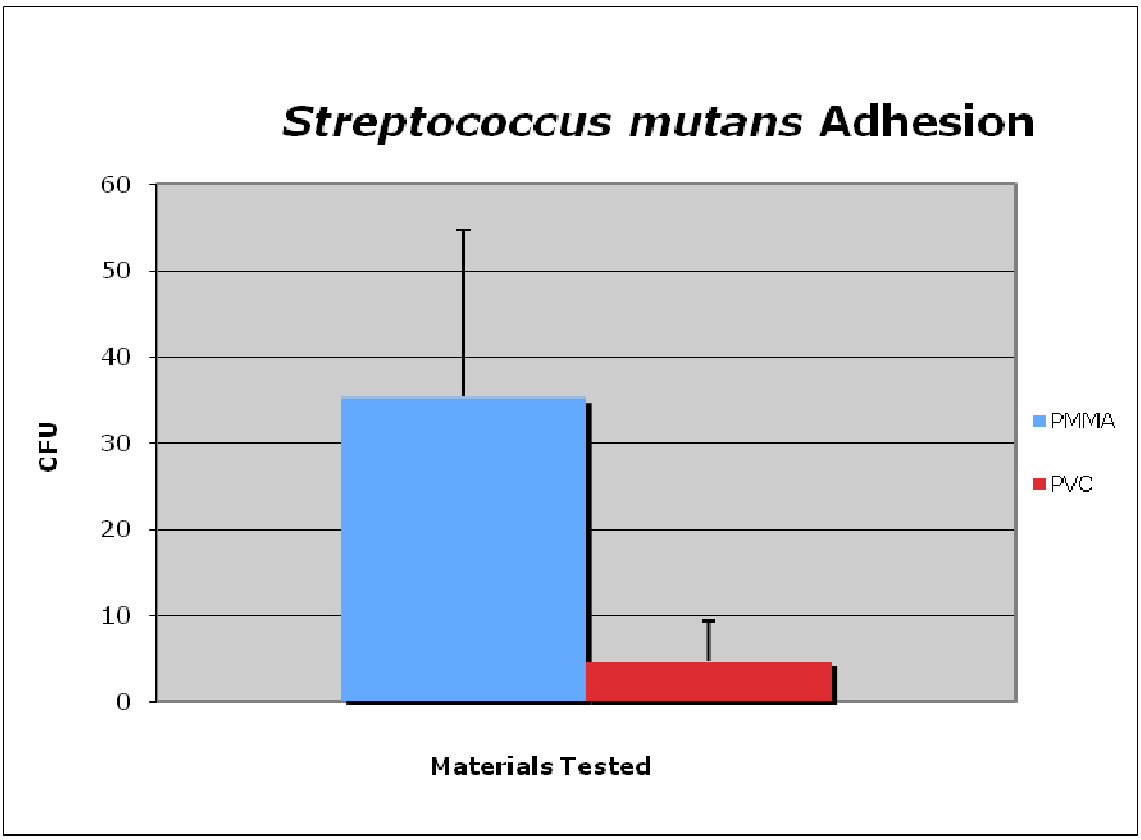

Figure 11: CFU of *Streptococcus mutans* adherent to PMMA and PP. Adherence was charted for comparison of SM at 10^{-4} dilutions. CFU's for triplicates were averaged for PMMA and PP. Overall PMMA had an increased amount of SM adherent compared to PP, calculating to roughly a 7-fold difference.

Figure 11 represents the adherence comparison of SM at 10^{-3} dilutions. CFU for triplicates were averaged for PMMA and PP, Overall, PMMA had a 7-fold greater amount of SM adherent bacteria compared to PP.

4.4 Statistical Analysis of Cultured Adhesion

LA was tested for the ability to adhere to polymethyl methacrylate (PMMA) or plastic vacuum formed material (PP) *in vitro*. Five (5) ten—fold serial dilutions were performed in a total volume of 10 ml of solution (MRS). The measurements

at dilutions 10^{-2} and 10^{-3} were found to be too numerous to count (TNTC). Analysis of the assay results demonstrated the average CFU for *L. acidophilus* adherence to PMMA (31.0 CFU ± 6.2; standard error (S.E.), *n* = 3) measured at the 10^{-4} dilution was three times greater than to PP (10.3 CFU ± 2.9 S.E., *n* =3). Although these results demonstrate the comparatively higher adherence of LA to PMMA than PP, the range of CFU was large enough that a statistical analysis using students' *t* test revealed these averages were not significantly different, *p* = 0.09 (one-tailed, unequal variance).

Streptococcus mutans (SM) was also tested for the ability to adhere to PMMA or PP *in vitro*. As previously described, five serial dilutions were performed using the appropriate media (TSB). Analysis of the assay results in table 2 demonstrate the average CFU. Similar to LA, the measurements of SM CFUs at the 10^{-2} dilution were found TNTC. However, analysis of the assay results demonstrated the average CFU for SM adherence to PMMA (35.3 CFU ± 6.4; standard error, *n* = 3) measured at the 10^{-3} dilution was more than seven times greater than to PP(4.7 CFU ± 1.5 SE, *n* =3). Statistical analysis using the *students' t test* revealed higher adherence of SM to PMMA than PP that were significantly different, *p* = 0.05(one-tailed, unequal variance).

Comparisons of both species' adherence to PMMA and PP was done for those samples with countable colonies (30-300 CFU). A three fold difference in adhesion of PMMA compared to PP existed for both bacterial species. Statistical analysis using a students T-test revealed higher adherence of SM was significantly different P = 0.0031, while LA was not P = 0.1350.

4.5 XTT Assay Testing for Media and Cell Number

The XTT assay was used to evaluate the number of adhered bacteria to the materials by measuring total bacterial cell metabolic activity. Initial experiments were performed to ascertain if the XTT was capable of measuring bacterial metabolic activity and to determine the cell number necessary to obtain reproducible results. Initial tests were performed to determine the number of cells that would not saturate the XTT color change but were high enough to give a range of values with differing concentrations of cells. Ten-fold increasing concentrations of SM and LA were placed directly into a 48 well plate to determine the number of cells needed to get a reliable reading for XTT, without saturating the colorimetric scale for absorbance. The XTT reagent was then added and the absorbance at 450 nm was monitored every hour. At 4 hours of incubation, absorbance readings were first clearly seen at $1X10^6$ CFU for both bacterial species (Figure 12). The inherent differences in the TBS and MRS media color led us to also test both bacterial species in PBS to determine if this colorimetric assay would be affected by background media color. SM's TSB media was light in color and did not interfere with the colorimetric readings. SM did not tolerate being in the PBS and gave poor metabolic readings in PBS and therefore SM was kept in TSB media for all incubations. LA's MRS broth was too dark and interfered with the colorimetric assay, therefore PBS was used for these incubations.

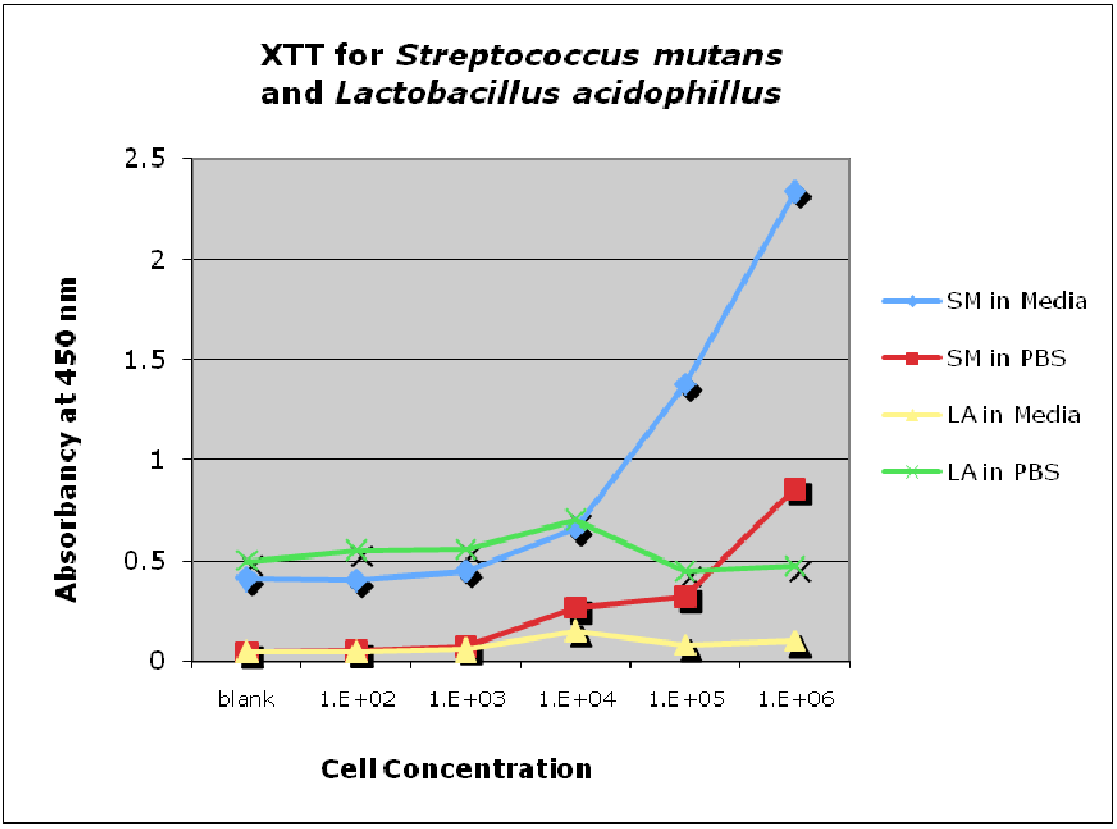

Figure 12: XTT for Cell Number and Media Type:
LA and SM both showed positive activity at $1X10^6$ cells per well. This value was taken as the absolute minimum number of cells needed to be accurately read by an XTT assay. In addition, media differences between bacterial cell lines showed the following. SM's TSB was light in color and did not affect the absorbance readings. SM did not grow well in PBS and therefore did not give us a reading. LA's media of MRS broth was too dark get an accurate reading for XTT. LA when substituted with PBS improved absorbance readings.

4.6 Adherence Testing by XTT Assay

The XTT reagent was used to test bacterial adhesion to our materials by indirectly determining cell number based on the bacterial metabolic activity. Bacterial cells were plated at a density of $3X10^8$ cells per well for both bacterial

species. The materials were incubated with or without saliva for one hour, followed by two hours of incubation with the bacteria for adhesion. The discs were then removed from the culture media, rinsed 3 times in PBS, and placed into new wells containing PBS for LA and TSB for SM. The XTT reagent was then added and the absorbance monitored at 450 nm, every hour up to 4 hours. Experimental controls consisted of media only and a sample that represented carry over (disc briefly dipped and then rinsed). Carry over was used to show the material or liquid's inherent ability to attract bacteria, which can lead to a background level of bacteria in the results. Bacteria were adhered to PMMA with and without saliva to determine saliva's effect on adhesion for each particular bacterial species. In this study, saliva was determined not to significantly affect adhesion. When comparing the XTT results of both materials pre-treated saliva, it is evident that PMMA had more bacterial adhesion than PP for both species examined (Figures 13 and 14). The amount of bacterial carry over due to liquid cultures attraction to the material is significantly greater that the no bacteria control but much less than the absorbance detected in adhered samples (Figure 13). These results led to future methodology changes incorporating an increased number of PBS washes in our protocol. Saliva demonstrated little change in bacterial adhesion levels and PMMA is more adherent than PP (Figure 13). The results of SM adhesion are shown in Figure 14. Improved methods allowed us to decrease the level of carry over bacteria to readings similar to that of media alone, indicating most carry over was due to liquid attraction to the disc. Saliva also showed little effect on SM's ability to adhere to PMMA. When both materials

were compared, PP demonstrated less adherence of SM than that of SM to PMMA.

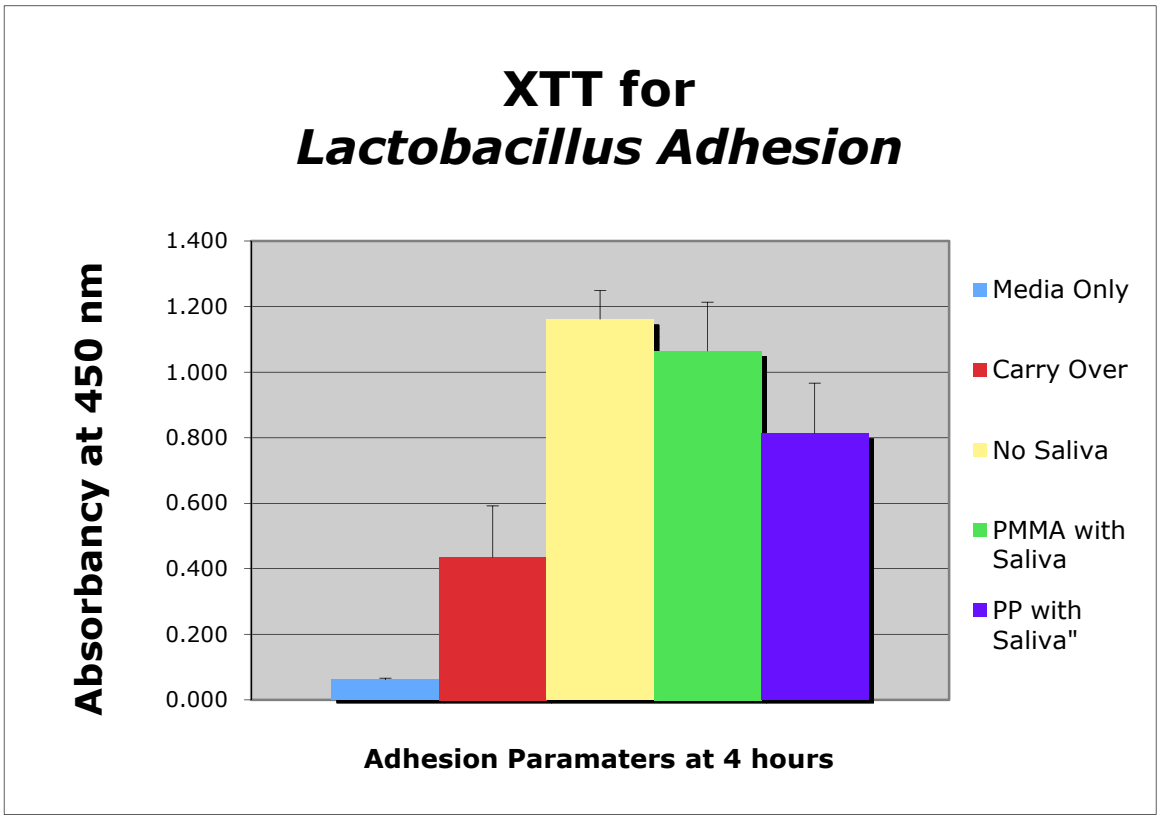

Figure 13: XTT Adhesion of *Lactobacillus acidophilus*
Discs of PMMA and PP were treated with or without saliva for one hour, and then incubated in LA culture for 2 hours. Discs were then rinsed and placed into a new well of PBS for the 4-hour incubation with XTT reagent. Plates were read at 450 nm and absorbance levels used to compare adhesion levels. Results show carry over is relevant to the methods and relates to hydrophilic interactions, saliva has little effect on adherence and adhesion of LA is decreased in PP compared to PMMA. Error bars represent the difference in OD for triplicates.

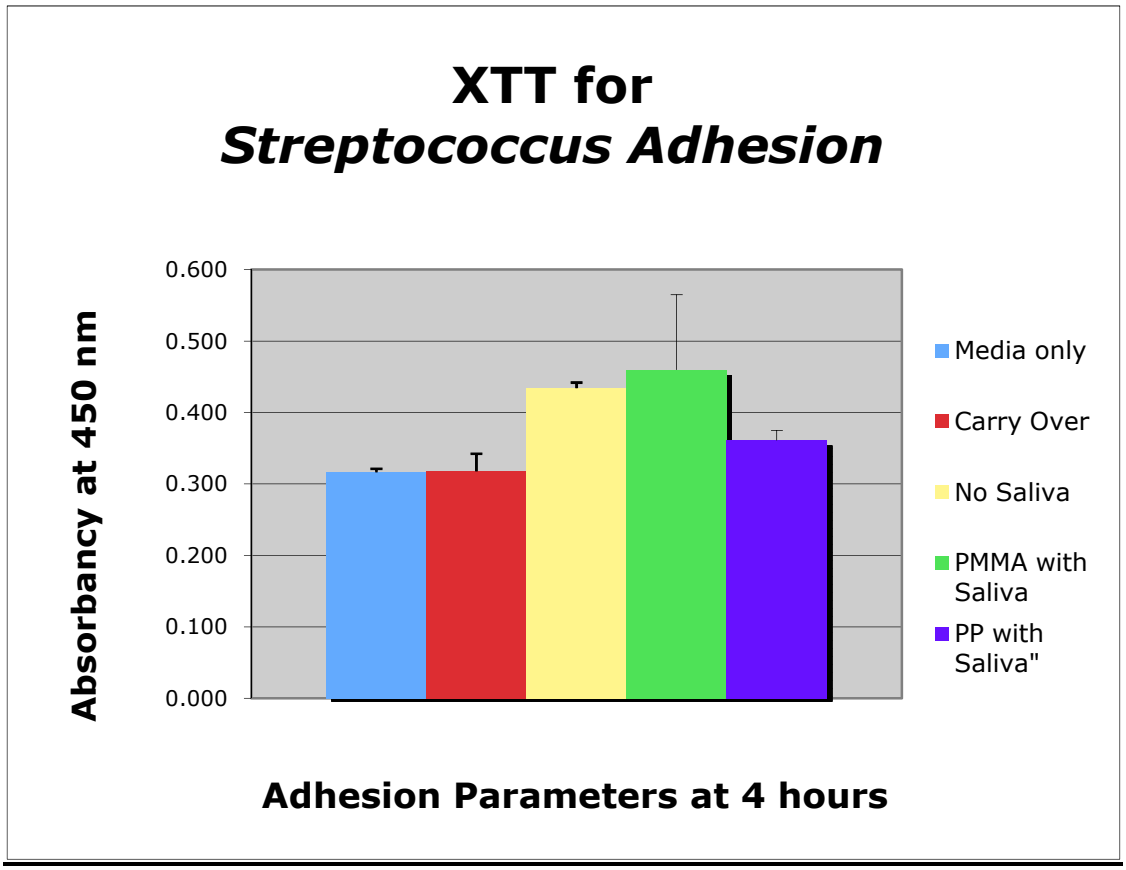

Figure 14: XTT Adhesion of *Streptococcus mutans*
Discs were treated with or without saliva for one hour, and then incubated in culture for 2 hours prior to a 4-hour incubation with XTT reagent. Plates were then read at 450 nm and absorbance was used to compare adhesion levels. Results show carry was less significant with changes in method, saliva has little effect on PMMA adhesion and bacteria were less adherent to PP than PMMA. Error bars represent the difference in OD for triplicates.

4.7 Statistical Analysis for XTT Adhesion Assay

Lactobacillus acidophilus (LA) adherence to PMMA or PP from *in vitro* adhesion assays was tested for metabolic activity and proliferative activity using the XTT assay. Analysis of the XTT metabolic profile of LA adherent to PMMA at four (4) hours revealed an average OD of 1.065 ±0.049 in absorbance (SE, n=3); nearly

thirty percent higher than PP, which averaged 0.814 ± 0.051 in absorbance (SE, n=3). Statistical analysis using t-distribution revealed this difference was statistically significant, p=0.04.

Streptococcus mutans (SM) adherence to PMMA or PP from the *in vitro* adhesion assays was also tested for metabolic activity and proliferative activity using the XTT assay. Analysis of the XTT metabolic profile for SM adherent to PMMA at four (4) hours revealed an average of 0.460 ± 0.03 in absorbance (SE, n=3), which was 27% higher than PP, which averaged 0.361 ± 0.004 in absorbance (SE, n=3). Statistical analysis using t-distribution revealed this difference was not statistically significance, p=0.122.

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Discussion of Results

The goal of this research was to decipher if any difference existed in the adhesion of SM and LA on two different orthodontic materials, PMMA and PP. Bacterial adhesion studies were performed on discs treated with or without saliva, prior to bacterial adhesion by SM and LA. After adhesion, the discs were rinsed in PBS and recovered cells were plated to determine the CFU of adhered bacteria on both materials. All adhesion studies were then repeated using XTT as an indicator of the number of adhered bacteria by measuring the bacterial metabolic activity.

The materials were not polished in order to study the materials inherent ability to attract and adhere bacteria, rather than measuring the effect of polishing the materials on bacterial adherence. Unpolished PMMA, used in our testing, would represent either the intaglio surface or PMMA that overtime has lost its original smoothness from normal wear. Often the original material polish is changed by chemical cleansers, brushing and the average wear and tear of the appliance. Although discs were submerged into bacterial cultures, only the top surface of the disc had bacteria firmly attached. Although the entire disc was swabbed, few bacteria existed on the bottom of the disc that rested on the floor of the well. This was ascertained through initial bacterial staining attempts that proved unreliable to document the number of adhered bacteria.

Our study of bacterial concentrations led us to determine that an original concentration of $3X10^8$ CFU per well was sufficient enough to accurately test adherence to the different materials. This concentration was similar to the concentration of bacteria used in other studies and therefore was used with confidence (Hahnel,S. 2008). Despite both bacteria being facultative, both cultures were grown in aerobic conditions due to convenience and the lack of anaerobic chambers for growth. While other similar studies utilized a tri-gas incubator or other anaerobic conditions, American Tissue Type Culture (ATTC) which provided the cultures, verified that both bacteria can be grown in aerobic laboratory conditions. It is possible that under preferred conditions of an anaerobic environment the bacterial cells may grow more rapidly, but our study more closely matches the aerobic environment the materials are routinely exposed to.

Multiple quantification methods were originally attempted for enumeration of adhered bacteria. Unfortunately, bacterial adherence was visible, but was easily removed during fixation and subsequent direct bacteria staining methods. Although differences in adhesion were initially visible to the naked eye, after PBS rinses those differences were much less obvious. This indicates a difference between the biofilm formed and strongly adherent bacteria. Multiple rinsing methods were attempted which ranged from vigorous rinsing to suction, but in the end, it seemed that the most reliable and precise technique was to dip the disc into PBS three times prior to quantification. This rinsing method is similar to other published procedures. As far as the material's properties, visually the

VFR's PP was much smoother, somewhat like a polished surface when compared to the PMMA. The VFM's edges were rough at the cutting edges, but still overall lacked the roughness visually seen in the acrylic PMMA discs. Of the discs used in experiments, a conscious effort was made to only use those discs free of visual defects. Polishing of the discs was not done, so that the results would render information about the retainer materials themselves, not the effects of different surface treatments.

Controls were used to test the validity of our methods and consisted of media and a carry over well. The carry over, which was a one sec. dip into the bacterial suspension prior to the PBS washes, served to demonstrate if any bacteria were transferred due to the polarity of water and its attraction to the disc after washing, rather than adhesion. Although not originally studied, it became evident that droplets of liquid remained attached to the discs after three PBS washings and the concern was that we were measuring the bacteria in those droplets, even though they were not strongly adherent to the materials. The carry over well was used to demonstrate that there was a minimal amount of bacteria transferred within these droplets. The background contamination was apparent when adherence was studied for LA. This was most likely due to the hydrophilic interactions between the bacterial medium and the disc. Fortunately this adherence was not significant when compared to the levels seen by either of the materials when incubated for 2 hours with the bacteria. After carry over was detected with the LA in initial experiments, our methods were improved by increasing the number of washes in PBS to 3 times and by gently touching the

side of the discs to the sterile well wall to draw off and decrease liquid drops from the disc. This resulted in a decrease in carry over seen for SM studies, matching closely to that of the media only well.

Saliva's influence on bacterial adhesion to PMMA was tested for both bacterial species. This was performed to demonstrate saliva's influence in bacterial adhesion because saliva's effects are thought to be bacteria specific. For both bacteria, negligible differences were evident in adhesion between those discs pre-treated with or without saliva. LA showed a slight increase in adhesion, where SM showed a very slight decrease in adhesion with saliva. Most research suggests that even the additional bacterial adhesion promoted by the presence of saliva, is diminished with time. This is because any additional binding sites provided by salivary proteins, have been occupied and with time contribute no additional adhesions sites after initial binding sites are bound. Published research clearly demonstrates that although the influence of saliva is very much bacteria species dependant, with elapsed time saliva, has less of an influence on binding (Ahn.S.J. 2002; Ahn.S.J. 2008).

The ultimate goal of this study was the comparison of bacterial adherence to PMMA and PP materials coated with saliva after incubation with two different oral bacterial species. Adhesion studies were conducted to determine the amounts of viable bacterial cells attached to the different materials. With both bacterial species, attachment was increased for PMMA compared to PP. Because only a subset of the data was able to be quantified due to CFU density on the plates, statistical significance was difficult to prove due to the large

variability between CFU for replicates. When considered in total, PMMA clearly demonstrated more bacterial adherence for both species tested. However, statistically significant difference in attachment was only found for SM. Overall both bacteria showed greater adherence to PMMA than PP, with LA resulting in a 3-fold difference and SM a 7-fold. When looking at those samples in which colonies were of optimal range for comparison (30 to 300 CFU), it was still evident in Table 2 that there was roughly a three fold increase in adherence for PMMA compared to PP for both bacteria, with significance being found for *Streptococcus mutans* and not *Lactobacillus acidophillus*. This could be due to a large range of colony counts between cultured triplicates. This is most likely due to the bacterial and media conditions of each particular bacterial culture, since values for each particular triplicate were consistently high or low in number.

When using the XTT assay to infer the number of attached bacteria, the results showed more bacterial adhesion to PMMA than PP for both bacteria. Similar to the results obtained for the CFU analysis, saliva did not significantly alter the bacterial adherence. However, a statistical significant difference in adhered bacteria was only found for LA in PBS. Although the XTT assay served as an important component of our study to confirm our previous CFU results, the sensitivity of this technique leaves room for error. The XTT assay was influenced by several factors independent of bacterial cell number that may have contributed to larger variations in results. It became apparent that the XTT assay is extremely sensitive to cell number as well as influenced by the background absorbance of the bacterial specific media and inherent properties of the specific

bacteria. The issues concerning the color of the culture media for the colorimetric XTT assay were addressed by changing LA's medium to PBS during incubation, however this only partially improve our results with XTT for LA. These results led to concerns that the turbidity of cell suspensions were also affecting our results. Unfortunately, our attempts to avert this by centrifuging and pelleting the bacterial suspension and only using the supernatant for readings were inconclusive. Therefore, all solutions were tested at four hours in their respective solutions, TSB for SM and PBS for LA. Another concern with the XTT assay was the observation that the XTT reaction product did not seem to be completely soluble in LA. This was represented by an attempt to pellet the bacteria cells and assay the supernatant. The bacterial pellet retained a noticeable orange pellet indicating that the soluble reaction product was not being released by LA. This issue of the reagent being insoluble and trapped within the bacterial cells could explain why absorbance readings for the XTT assay were not clear. Past studies with the XTT reagent also found that certain bacteria resulted in XTT colorimetric products that was not completely soluble, which contributes to inaccuracies despite consistent differences in adhesion values(Kuhn,D.M. 2003). The XTT assay is also difficult to utilize to conduct growth testing or adherence assays over time, due to the saturation of the test with a large number of cells. If the initial number of adhered bacteria is too low, the XTT assay will be insignificant due to lack of color development. Therefore, this assay's sensitivity allowed us only a vague representation of differences in attachment levels of the two bacterial species. In addition, the intra assay variability was quite large as

evidenced by the large error bars between the cell concentrations of triplicate wells. Despite consistent absorbance readings indicating adhesion was higher for PMMA than PP for both bacterial species, overall the results were overshadowed by the difficulty in obtaining reproducible measurements with the XTT assay. Published studies using the XTT reagent have suggested that this reagent is not to be used for cell concentration determination nor for the comparison between cell suspensions due different properties with certain cells over others. Therefore, although it can be a valuable experimental tool to determine viability and therefore number of adherent cells, it is not accurate nor reliable for all bacterial cells types at the same level (Kuhn,D.M. 2003). In general, the XTT assay for LA and SM adherence indicated that both bacteria were less adherent to PP than PMMA, with LA showing 30% less adherence and SM 27%, respectively.

In conclusion, we have clearly demonstrated by both a quantitative assessment of the number of CFU and a qualitative assessment of the number of bacteria present via metabolic activity that both bacterial species investigated adhere more strongly to PMMA than to PP. Some variability in the methods coupled with a small sample size of quantifiable CFU prevented an accurate representation of the significance of this adherence.

5.2 Recommendations for Further Study

While our overall results clearly demonstrate an increased adherence of bacteria to PMMA versus PP, future research should focus not only on *in vitro*

but also *in vivo* studies on the bacterial attachment of thermoformed polymer retainer material compared to the components of a traditional Hawley retainer. In addition to caries bacteria, those organisms involved in periodontal disease and fungal diseases such as candidiasis should be investigated. Because specific bacteria have different adherence properties and differences exist as to whether saliva affects this binding, its crucial to understand which retainers are best for certain patients. In addition, patients with xerostomia or who are medically compromised could benefit from knowing that their retainer will not worsen their condition. Split mouth studies involving retainers consisting of both materials, would allow a direct comparison of the two retainers inherent bacterial adherence properties in a patient population and how that adherence may change with time. These studies were conducted to assay for adherent bacteria that withstood 3 moderate rinses with PBS. The question must be addressed in the future is what level of bacterial adherence does this represent. In the future, different rinse techniques, such as a vigorous rinse could be attempted to discover the amount of bacteria that are strongly bound and compare it to the amount of bacteria easily removed by shear forces. Vacuum suction of the bacterial suspension coupled with rinsing with PBS could further lessen the amount of cell carry over from hydrophilic interactions to the discs giving accurate results. Cotton swabbing to recover the adhered bacteria, although known as a reliable method for culture analysis, can be worrisome, since its fibers could trap bacteria resulting in an underestimation of adhered bacteria. Although the samples are centrifuged to pellet the recovered bacteria, it is unclear if we are separating and

removing all of the bacteria from the cotton swab. Currently this method appears adequate because all samples experience the same procedure, making any bacteria loss consistent throughout all samples. Cell scraping could be attempted, but in our study the size of the material sample was too small for this type of maneuvering. Additional methods such as enzyme-linked immunosorbent assay (ELISA) could overcome the issues associated with swabbing for culture analysis, but issues with bacteria sticking to wells means the samples must be redistributed into fresh wells for an accurate measurement. Once again, although cell adherence to all wells would be constant, cell division is so rapid that many tests become too saturated to read, making spread plating and CFU counting the most accurate method for determining which material is most adherent. Another method for testing could be using fluorescence or radiolabeling to measure cell adhesion and viability. Furthermore, future studies could measure not only adherence, but also how bacterial growth is affected by the salivary pellicle and its behavior over time as attachment sites decrease. It is possible that initial adhesion is irrelevant, and the overall size or position of the appliance is more representative of the nature of attachment.

Improving the XTT assay or perhaps using another metabolic assay, could improve sensitivity allowing less bacterial cells to be used for accurate readings while not saturating the assay. Currently we were able to accomplish this for the two-hour adhesion studies, but growth studies would require a test with a higher specificity and sensitivity. Additionally, future studies focusing on longer times of adhesion and the assaying for the relative strength of that adhesion, could

provide additional insight and be beneficial for oral hygiene protocols. Once a more standardized test, with improved methods is available, better comparisons can be made between the two materials and differences in bacterial attachment, as well as the reason for it.

Additional knowledge would be gained by using scanning electron microscopy or the development of an improved staining method for visualizing the attachment patterns of specific bacteria and its effect on overall number. Visual surface topography of both materials would allow us to see, if certain portions of the material are more adherent than others. Areas such as the edges of a VFR which sit along the periodontal sulcus or the traditionally roughened intaglio surface of a Hawley retainer which rests on the palate or gingival floor may differ in adhesion properties from those found elsewhere. Testing could then be extended to examine polished versus unpolished surfaces to more accurately replicate how the material is represented in the mouth. In addition, studies could potentially look at the different roughness and surface energy values for these particular materials, to gain a better understanding of just what is driving bacterial adherence.

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