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A biofilm-based aging model for testing degradation of dental adhesive microtensile bond strength

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A BIOFILM-BASED AGING MODEL FOR TESTING DEGRADATION OF DENTAL ADHESIVE MICROTENSILE BOND STRENGTH

by

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MASTER'S THESIS

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To Mom and Dad and Anshul for your unconditional love, support and encouragement

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ABSTRACT

The objective of this work was to develop a short-term, clinically simulative, biofilm-based aging/storage model for lab testing of newer dental adhesives in order to predict their long-term performance. To do this we tested the hypothesis that 15 days of biofilm challenge with cariogenic bacterial species, *Streptococcus mutans (SM)* and *Streptococcus sobrinus* (SS), would produce similar or a greater reduction in microtensile bond strength (μTBS) of dental adhesives as compared to a standard 6 months of water storage (WS).

Thirty-one molars were flattened to dentin, restored using Optibond-FL adhesive and Z-100 dental composite, sectioned and trimmed into four dumbbell-shaped specimens and randomly distributed according to aging conditions (n=31): A) Water storage for 6 months, B) Water storage for 5.5 months + *S. mutans*-biofilm challenge for 15 days, C) *S. mutans*-biofilm challenge for 15 days and D) *S. sobrinus*-biofilm challenge for 15 days. Specimens were gripped centrally with respect to the test axis with a non-gluing passive gripping device. Microtensile bond strength testing was performed using a Zwick Material Testing Machine at a crosshead speed of 1 mm/min and failure modes were classified using light microscopy.

Mixed model ANOVA and Weibull regression analysis revealed that the type of storage condition significantly affected the microtensile bond strength ($p<0.0001$). Mean microtensile bond strength observed within group A (49.69 ± 15.53MPa) was significantly higher than those in groups B (19.26 ± 6.26MPa), C (19.92 ± 5.86MPa) and D (23.58 ± 7.88MPa). Also, microtensile bond strength obtained with group D was significantly greater than that with groups B and C, while no difference was seen between

the latter two groups. Chi-square statistical analysis indicated that specimens from groups B (74.2%), C (83.9%) and D (80.6%) were more likely to have cohesive failures in dentin than specimens from group A (54.8%).

Within the limitations of the study, it can be concluded that 15 days of *Streptococcus mutans*- and *Streptococcus sobrinus*- based biofilm challenge produced more reduction in microtensile bond strength of dental adhesive than 6 months of water storage and appear to be a promising *in vitro* accelerated aging model.

PUBLIC ABSTRACT

In order to evaluate the effectiveness of dental adhesives used in tooth restorations, one must mimic the usage of the adhesive material under conditions that simulate the oral environment. Of the many such aging conditions that are available, a 6-month water storage (WS) protocol is the most recommended. The objective of the present study was to see if a much shorter, clinically simulative bacterial challenge could be used as an effective aging method. Two bacterial species commonly associated with dental decay and restoration failure were tested, namely, *Streptococcus mutans* (SM) and *Streptococcus sobrinus* (SS). The study compared the effect of 6-month WS to that of a 15-day SM or SS challenge on the bond strength values of dental adhesive. Bond strengths were calculated at the end of the aging period by measuring the force required to separate the adhesive-tooth (dentin substrate) bonded specimens. The broken specimens were then evaluated under higher magnification to determine the location of failure.

Statistical analysis of the data indicated that 15-day SM or SS bacterial challenge produced more degradation of resin-dentin bonds resulting in lower bond strength values of the adhesive than 6-month WS. It was also observed that specimens exposed to bacteria were more likely to fail within the dentin substrate indicating greater dentin demineralization following the bacterial exposure.

Within the limitations of the study it was concluded that 15-day SM or SS bacterial challenge produced more degradation of dental adhesive than 6-month WS and appear to be a promising laboratory aging model.

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CHAPTER I: INTRODUCTION

Resin-based dental composites are being increasingly used as both direct and indirect types of restorations, for anterior and posterior teeth. The introduction of dental adhesives, almost fifty years back, made it possible to bond composites to the enamel/dentin substrate. These bonding agents have also facilitated concepts like "minimally invasive dentistry" and "repair instead of replacement" that have become the guiding principles of present day dental practice (Van Meerbeek, De Munck et al. 2003, Van Meerbeek, Peumans et al. 2010, Liu, Tjaderhane et al. 2011, Heintze 2013). The success of the composite restorations relies on the durability of the adhesive joint.

Researchers are concerned about the mounting number of failures and replacements of composite restorations (Deligeorgi, Mjor et al. 2001, Manhart, Chen et al. 2004, Bernardo, Luis et al. 2007). The degradation of the adhesive-enamel/dentin joint is one of the main reasons for failure of composite restorations and is considered the weak link (Donmez, Belli et al. 2005, Spencer, Ye et al. 2010, Spencer, Ye et al. 2014). Manufacturers are constantly introducing new adhesives in an effort to simplify the application technique and to improve the bonding properties to enamel and dentin. It is imperative to test the bonding properties and validate the manufacturer's claims with sufficient scientific evidence before using the bonding agents in clinical practice.

Like in any other field, it is common knowledge that clinical trials are more definitive in evaluating the durability of dental adhesives. But they take more time and are expensive. Therefore, laboratory methods such as bond strength testing and margin analysis have been devised to evaluate the performance of dental adhesives. Additionally, to simulate the conditions of the oral environment, *in vitro* 'aging techniques' like mechanical loading, thermocycling and water storage have been used. Bond strengths obtained using an *in vitro* aging method correlated well with the results of clinical trials

(Van Meerbeek, De Munck et al. 2003, De Munck, Van Landuyt et al. 2005, Van Meerbeek, Peumans et al. 2010). However, these aging techniques have disadvantages that limit their use. As a minimum, 6 months of water storage has been recommended for optimal aging of dental adhesives in a laboratory setting (Burrow, Tagami et al. 1993, Armstrong, Keller et al. 2001b, ISO/TC 2015). Thermocycling is time consuming and there is no consensus among researchers as to the number of cycles or protocol necessary for adequate aging of adhesives (Morresi, D'Amario et al. 2014). Also, these aging methods only challenge the hydrolytic and mechanical stability and fail to evaluate the enzymatic stability. Therefore, in this competitive era of dental bonding agents where time is of the essence we require a laboratory aging method that can expedite the aging of resin-dentin interface and aid in evaluating the long-term bonding properties of dental adhesives.

Purpose of the Study

The objective of this work is to develop a short-term, clinically simulative, biofilm-based aging model for lab testing of newer dental adhesives. To do this we compared the effect of two weeks exposure to a *Streptococcus mutans* or *Streptococcus sobrinus* cariogenic bacteria-based biofilm and 6 months of water storage on microtensile bond strength (μTBS) of dental adhesive. A few studies have been done to evaluate the effect of biofilm on resin composites and adhesives (Bourbia, Ma et al. 2013, Mutluay, Zhang et al. 2013, Borges, Kochhann et al. 2014, De Carvalho, Puppin-Rontani et al. 2014, Li, Carrera et al. 2014). However, none of them have evaluated the biofilm-assisted degradation as a potential *in vitro* aging technique to complement the microtensile bond strength testing.

CHAPTER II: LITERATURE REVIEW

Dental Caries

Dental caries is a pathological process, a result of the interaction between cariogenic bacteria and fermentable carbohydrates on tooth surfaces over time. While most bacteria within coronal plaque are saccharolytic, *Streptococcus mutans* and *Lactobacilli* are especially acidogenic and aciduric organisms that are commonly associated with dental caries. These bacteria metabolize carbohydrates for energy and in the process form organic acids as the by-product. With time, the acids accumulate, and demineralize the tooth surface when the pH dips below 5.5 for enamel and 6.2 for dentin. If there is no intervention, the process of demineralization ultimately results in cavitated defects in the teeth. The carious portion is excavated and replaced with artificial restorative materials, such as amalgam, resin composites or glass ionomers to restore the form and function of the tooth (Heymann, Edward J. Swift et al. 2013). Resin composites have become increasingly used because of their superior aesthetics and adhesive properties. The growing concern about 'mercury toxicity' has also prompted the use of resin composites (Spencer, Ye et al. 2014).

Resin Composites

Resin composites are tooth-colored restorative materials. They are composed of polymeric matrix, filler particles, organo-silane coupling agent for binding the fillers to the matrix, and an initiator-activator system for carrying out the polymerization (Ferracane 2011). Resin monomer 2,2-bis[4-(2-hydroxy-3-methac-ryloxypropoxy)phenyl]-propane (Bis-GMA) along with diluents such as triethylene glycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA) or ethoxylated bisphenol-A-dimethacrylate (Bis-EMA) are most commonly used to form the polymeric matrix (Ferracane 1995, Peutzfeldt 1997, Chen 2010, Ferracane 2011). Filler particles

including quartz, silicon dioxide and radiopaque silicate glass containing barium, strontium, zirconium or aluminum are used to strengthen resin composites and to reduce their shrinkage and thermal expansion (Ferracane 1995, Chen 2010). Visible light-activated, camphoroquinone-tertiary amine based initiator-activator system is most commonly used for carrying out the free radical addition polymerization of resin composites. There can also be 'dual cured resins' that have both light-cured and an additional chemically-cured component (Stansbury 2000, Ferracane 2011). The composites can be classified based on their filler size as: macrofill (10-50μm), microfill (40-50nm), nanofill (5-100nm), minifill (0.6-1μm) and midifill (1-10μm) (Ferracane 2011). However, composites do not wet nor adhere well to the tooth surface and therefore require an additional layer of dental bonding agents that hold the composites to tooth substrate (enamel/dentin).

Dental Bonding Agents

Bonding agents are defined as the material applied between two surfaces to join them together, resist separation, and help transmit occlusal load. This process is called adhesion and the result of such interaction is termed a bond. Bonding of dental adhesives to tooth substrate (enamel/dentin) involves dissolution of calcium-phosphate molecules from these mineralized tissues. This is followed by infiltration and in-situ polymerization of adhesive resin within enamel pits, opened dentinal tubules and around exposed collagen fibrils. This process is called 'hybridization' and the resulting zone is the hybrid layer (Nakabayashi, Kojima et al. 1982, Pashley, Tay et al. 2011).

Classification of Dental Bonding Agents

Bonding agents can be classified according to their application strategy as 3- and 2-step etch-and-rinse, 2- and 1-step self-etch and universal bonding techniques.

The etch-and-rinse technique

This technique involves application of acid etchant, primer (adhesion-promoting agent) and the bonding agent. The 2-step version combines the primer and bonding agent together. The etch-and-rinse approach yields the most stable bonding to enamel and is considered to be the 'gold-standard' (Van Meerbeek, Peumans et al. 2010). However, the bonding to dentin is weaker than the bonding to enamel because the aggressive etchant leaves 'hydroxyapatite-depleted' collagen in dentin that is difficult to infiltrate (Van Meerbeek, De Munck et al. 2003). Also, this protocol is technique sensitive due to the number of steps. This has led to the evolution of self-etch protocols with fewer steps.

The self-etch technique

This technique does not have a separate step of etching and rinsing. Instead, this technique incorporates the etchant into the primer (2-step) or all components in one (1-step). It can be further divided based on the pH of the self-etching primer as 'strong' (pH <1), 'intermediate' (pH 1.5), 'mild' (pH = 2) and 'ultra-mild' (pH ≥ 2.5) (Van Meerbeek, De Munck et al. 2003, Van Meerbeek, Yoshihara et al. 2011). The fewer steps help in minimizing the chances of operator errors and decreases the application time (Van Meerbeek, De Munck et al. 2003). However, the 'mild' and 'ultra-mild' self-etch adhesives do not produce adequate demineralization and result in poorer bonding to enamel than obtained with etch-and-rinse adhesives. So, selective etching of enamel margins with a phosphoric acid etchant prior to the application of these adhesives has been recommended (Van Meerbeek, De Munck et al. 2003, Van Meerbeek, Yoshihara et al. 2011).

Universal bonding technique

As the name suggests these newer 'universal' adhesives can be applied using self-etch, selective enamel etching, or total-etch and rinse techniques. However, as they remain self-etch adhesives, selective enamel etching is advocated with universal adhesives to improve their bond strength to enamel (Hanabusa, Mine et al. 2012, Perdigao, Kose et al. 2014, Loguercio, de Paula et al. 2015, Rosa, Piva et al. 2015). Also, it has also been suggested that universal adhesives can be used with different substrates such as 'wet' or 'dry' dentin and silica-based glass ceramics or zirconia (Hanabusa, Mine et al. 2012, Chen, Niu et al. 2015, Loguercio, de Paula et al. 2015).

Bonding to Different Tooth Substrates

Micromechanical bonding to enamel has excellent longevity because it is primarily made of inorganic calcium-phosphate crystals (95 - 98 weight%) with only 4 weight% of water and 1 - 2 weight% organic material. On the other hand, organic material (18 weight%), mainly type I collagen and water (12 weight%) are substantial component of dentin. The inorganic content of dentin is only 70 weight%. This poses a challenge for bonding of hydrophobic resin materials (Summitt, Robbins et al. 2006). Therefore, the concept of chemical bonding in addition to micromechanical interaction between resin monomers and organic content (collagen) of dentin has gained popularity (Van Meerbeek, Peumans et al. 2010, Van Meerbeek, Yoshihara et al. 2011).

Clinical Challenges and Bond Durability

Earlier, resin composites exhibited material-related problems such as poor wear resistance, color stability and inferior handling properties. However, most of these weaknesses were overcome with advancement in material sciences (Sarrett 2005). For example, introduction of smaller sized filler particles improved the polishability, polish

retention and wear properties of resin composites (Ferracane 2011). Presently, secondary caries and restoration fracture are cited as the main reasons for the replacement of composite restorations (Manhart, Chen et al. 2004, Sarrett 2005, Ferracane 2011). With time, there is loss of the marginal seal at the adhesive-tooth interface, which leads to infiltration of oral fluids, bacteria and their by-products along these gaps. This is manifested clinically as hypersensitivity, marginal staining and secondary caries (Heintze 2007, Spencer, Ye et al. 2010). Restoration fractures seen with resin composites could be due to flaws in the cavity design, compromised tooth structure or weak adhesive bonding (Manhart, Chen et al. 2004, Sarrett 2005). The compromise in the durability of the adhesive-tissue interface can be attributed to the polymerization shrinkage of the resin material itself or to operator errors such as faulty composite placement and incomplete polymerization, or to the degradation of the adhesive bond (Heintze 2007).

Both the resin material and the dentinal collagen fibers of the adhesive zone/hybrid layer are prone to *in vivo* degradation. Hydrophilic and ionic monomers present in the bonding agents promote water sorption resulting in hydrolytic damage of collagen and 'plasticization' of the resins (De Munck, Van Landuyt et al. 2005, Liu, Tjaderhane et al. 2011). Furthermore, due to the presence of water, solvents or dentinal fluid exudates, demineralized collagen may not be completely infiltrated by the adhesive resins. These exposed collagen fibers are then subjected to degradative proteolytic enzymes such as matrix metalloproteinase (MMPs) and cysteine cathepsins that are present in dentin, dentin fluids and saliva. Acid etching can also activate these MMPs and cysteine cathepsins (De Munck, Van Landuyt et al. 2005, Spencer, Ye et al. 2010, Liu, Tjaderhane et al. 2011, Pashley, Tay et al. 2011, Tjaderhane, Nascimento et al. 2013).

Thus, it is evident that mechanical properties and chemical integrity of the adhesive-resin interface determine the durability of composite restorations. Clinical trials and laboratory tests have been used for testing the properties of new bonding agents

before they are used clinically. The following sections give an overview of important aspects related to testing of dental adhesives.

Clinical Trials

Retention rate of non-caries class V adhesive restorations are commonly used for evaluating the bond effectiveness of dental adhesives in a clinical setting (van Dijken 2000, Aw, Lepe et al. 2005, van Dijken, Sunnegardh-Gronberg et al. 2007, Peumans, De Munck et al. 2012). Clinical trials are the 'gold standard' for materials testing and provide the most accurate assessment of long-term bond effectiveness (Van Meerbeek, De Munck et al. 2003, De Munck, Van Landuyt et al. 2005). However, there are limitations to these clinical studies that make them less popular. Clinical trials are difficult to standardize (Oilo 1993). The performance of dental adhesives in a clinical study is affected by multiple factors such as the patient's age, eating and oral hygiene habits, mechanical and thermal stresses in the oral cavity and other operator-related factors (De Munck, Van Landuyt et al. 2005). This makes it difficult to establish a cause and effect relationship between specific variables and the retention failure. Cost and time are other disadvantages of clinical studies. It takes almost 3-5 years (Van Meerbeek, De Munck et al. 2003) to obtain clinically useful data and by that time the adhesive might become obsolete and replaced by newer bonding agents. This defeats the purpose of undertaking the clinical trial.

Laboratory Tests

Laboratory testing overcomes some of the disadvantages found in clinical trials. They are relatively faster and easier to carry out. The impact of a particular variable can be studied while keeping the effect of other variables constant. Laboratory parameters of bond strength measurement, microleakage and microscopic evaluation of the adhesive-

tissue interface have been used for assessing the bonding efficacy of dental adhesives (Oilo 1993, Heintze 2013).

The International Organization for Standardization (ISO) developed a document, 'ISO/TS 11405:2015 Dental Materials – Testing of Adhesion to Tooth Structure'. This document gave specifications for substrate selection, storage and handling and essential characteristics of different laboratory tests. The methods included were tensile bond strength testing, measurement of marginal gaps and microleakage testing (ISO/TC 2015). Standardization helped in analyzing and comparing the data obtained from different laboratories.

The rationale for using bond strength testing was that stronger adhesion between the bonded surfaces would withstand higher environmental stresses and survive for longer duration (Van Meerbeek, De Munck et al. 2003). Bond strength is expressed in megapascals (MPa). It is calculated as the force (in Newton) per unit cross-sectional area (mm^2) required to break a bonded assembly with failure occurring in or near the adhesive interface (ISO/TC 2015). The fractured surfaces are analyzed under a microscope to study the debond pathway, which gives an indication about the weaker areas of the adhesive interface. The failure mode is classified as adhesive if the fracture is at the adhesive-dentin interface, or cohesive if the fracture is within the resin material or dentin substrate (Armstrong, Geraldeli et al. 2010).

The bond strength of the resin-dentin interface is assessed under 'shear' or 'tensile' types of forces, which are similar to the stresses applied in the oral environment. The tests were classified as 'macro' or 'micro' based on the bonded surface area being greater than or less than $3mm^2$, respectively (Van Meerbeek, Peumans et al. 2010). Macroshear- and microtensile- bond strength testing are the most commonly used methods in the literature and are summarized below:

Macroshear bond strength test

In this test, the bonded interface is exposed to forces working parallel to the tooth surface (Figure 1) and stressed until failure occurs (Oilo 1993). The substrate in figure 1 represents dentin and adherend refers to the body that is held by the adhesive (generally resin composite).

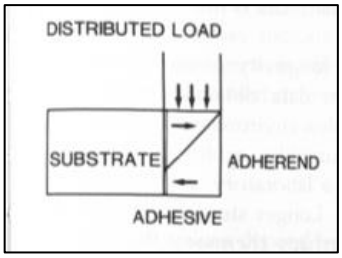

Figure 1: Shear bond strength testing

(As adapted from Oilo, G. (1993). "Bond strength testing--what does it mean?" International Dental Journal 43(5): 492-498)

These tests were popular because they were simple to perform. But shear bond strength testing required larger bonded areas, in the range of 3-5 mm^2. As a result, bond strengths of only 10-15 MPa could be tested. Beyond this range there were increasing numbers of cohesive failures (Pashley, Sano et al. 1995, Pashley, Carvalho et al. 1999). Furthermore, the newer adhesives surpassed these bond strength values, which warranted development of a different testing method. A microtensile test was introduced which was capable of measuring higher bond strengths (Sano, Shono et al. 1994, Pashley, Sano et al. 1995).

Microtensile bond strength test

The test was originally proposed by Kemper K and Kilian R (1976) to measure the bond strength of dental adhesives (Kemper K and Kilian R 1976). As illustrated in figure 2, the

bonded interface in this test is subjected to force operating at a 90^{o} angle to the tooth surface (Oilo 1993). The substrate in figure 2 represents dentin and adherend refers to the body that is held by the adhesive (generally resin composite).

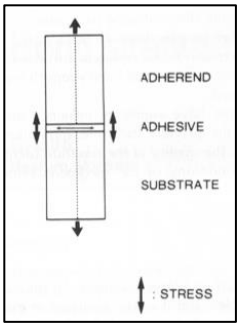

Figure 2: Tensile bond strength testing

(As adapted from Oilo, G. (1993). "Bond strength testing--what does it mean?" International Dental Journal 43(5): 492-498)

Advantages and limitations

Sano et al. (1994) and Phrukkanon et al. (1998) found an inverse relationship between the bonded surface area of dentin and the tensile strength of the adhesive material (Sano, Shono et al. 1994, Phrukkanon, Burrow et al. 1998). With the smaller bonded area ($\leq 1mm^2$), better stress distribution during loading may be possible and interfacial bond strengths higher than 25MPa and up to 65MPa can be measured (Pashley, Sano et al. 1995). Higher bond strengths can be explained by Griffith's defect theory according to which specimens with smaller cross-sectional area would have fewer defects resulting in higher bond strengths when tested under tension (GriffithAA 1920, Sano, Shono et al. 1994). Furthermore, multiple specimens could be obtained from each tooth for the microtensile bond strength (μTBS) testing (Sano, Shono et al. 1994) and there was better control of disparity between peripheral and central dentin specimens

(Van Meerbeek, Peumans et al. 2010). Sano et al. (1994) observed more adhesive failures and fewer cohesive failures in dentin with μTBS testing (Sano, Shono et al. 1994). The smaller surface area facilitated microscopic examinations of the failed bonds. The μTBS testing method also offered flexibility such that bonds made to small irregular surfaces could also be tested (Pashley, Sano et al. 1995). However, there are limitations to μTBS testing. The extra steps involved in specimen preparation after bonding make the microtensile testing method relatively laborious and technique sensitive (Van Meerbeek, De Munck et al. 2003). Also, there is the possibility of dehydration and damage with the smaller specimens used in these tests (Pashley, Sano et al. 1995, Armstrong, Geraldeli et al. 2010).

Gripping devices

Since the μTBS test relies on forces acting 90^{o} to the bonded surface, it is critical to maintain the alignment of the specimens (Oilo 1993, Armstrong, Geraldeli et al. 2010) during testing to avoid unequal stress concentrations. Active (Geraldeli's jig, Ciuchhi's jig) and passive gripping devices (Dircks device) have been developed to maintain the position of the bonded interface during μTBS testing. Active gripping devices use cyanoacrylate glue to attach the specimens to the surface of the jig (Armstrong, Geraldeli et al. 2010). However, the application of the glue, its detachment during testing and difficulty to clean the device afterwards is often problematic and produces errors (Soares, Soares et al. 2008). Passive devices are non-gluing and maintain orientation by constant contact between the 'neck' of the specimen and the gripping faces of the device. They have more reliable specimen alignment (Armstrong, Geraldeli et al. 2010). Raposo et al. (2012) found that the passive-gripping Dircks device had lower probability for operator errors and produced more uniform stress distribution at the adhesive interface when compared to the active-gripping Geraldeli's jig (Raposo, Armstrong et al. 2012).

Microtensile bond strength test specimens

Specimen preparation involves application of dental adhesive on the dentin surface of an extracted tooth. Incremental layering of resin composite follows the application of the adhesive layer. This assembly is then sectioned to obtain individual test specimens. Different specimen shapes have been used in μTBS studies like the rectangular slab, square stick, dumbbell, and hourglass (Phrukkanon, Burrow et al. 1998, Soares, Soares et al. 2008, Armstrong, Geraldeli et al. 2010, Raposo, Armstrong et al. 2012). Soares et al. (2008) studied the stress distribution in various specimens during μTBS testing using finite element analysis (FEA). They observed that dumbbell-shaped specimens had more uniform stress distribution when compared to rectangular and hourglass-shaped specimens (Soares, Soares et al. 2008). However, dumbbell-shaped specimens were found to be sensitive to defects introduced during trimming. There are increased areas of stress concentrations with the hourglass-shaped specimens and they tend to fail under lesser force (Armstrong, Geraldeli et al. 2010).

Correlation with Clinical Trials

Microtensile bond strength (μTBS) data for different adhesive systems were found to correlate with the retention rates of adhesive Class V restorations (Van Meerbeek, De Munck et al. 2003, Van Meerbeek, Peumans et al. 2010, Heintze, Thunpithayakul et al. 2011). Van Meerbeek et al. (2010) highlighted that the association was higher with 'aged' bond strength data and longer-term clinical trials (Van Meerbeek, Peumans et al. 2010). Thus, *in vitro* aging techniques have been commonly used in addition to bond strength testing to assess the durability of dental adhesives (De Munck, Van Landuyt et al. 2005).

In Vitro Methods for Aging

In the oral cavity, restorations are subjected to temperature changes, chewing load and chemical attacks by acids and enzymes. These conditions affect the bond strengths and durability of dental adhesives and should be taken into account when assessing the bonding properties of newer adhesives in a laboratory setting. Researchers use *in vitro* aging techniques like water storage, thermocycling, occlusal loading and exposure to salivary enzymes, which represent more clinically relevant challenges. We plan to compare water storage and biodegradation techniques of aging in the present study. The following sections give an overview of the literature related to these areas.

Water storage

Most studies have found significant reductions in bond strengths *in vitro* using long-term water storage (Gwinnett and Yu 1995, Armstrong, Keller et al. 2001b, Armstrong, Vargas et al. 2003, De Munck, Van Meerbeek et al. 2003, De Munck, Mine et al. 2011). It was established that 'at least 6-months' of water storage was required before any changes could be detected at the failure site of the resin-dentin interface. It was also observed that the failures were located at or near the interface (Burrow, Tagami et al. 1993, Armstrong, Keller et al. 2001b). Gwinnett and Yu (1995) found significant loss of resin-dentin bond strengths following 6 months of water storage when compared to 24hours of water storage (Gwinnett and Yu 1995). Armstrong et al. (2001) compared the microtensile bond strengths of dental adhesives after 30- and 150-days of water storage. They found that long-term water storage resulted in an increase in 'joint' failures when compared to the short-term water storage (Armstrong, Keller et al. 2001b). Also, a shift in the fracture pathway was observed within the joint from interphase between the top of the hybrid layer and adhesive resins to the bottom of the hybrid layer and dentin. It was postulated that the shift indicated that the bottom of the hybrid layer was the

'weakest link' for bond durability (Armstrong, Keller et al. 2001a, Armstrong, Keller et al. 2001b).

The decrease in bonding properties after water storage has been attributed to degradation of adhesive resins and/or collagen due to water sorption and hydrolysis (Gwinnett and Yu 1995, Armstrong, Keller et al. 2001b, De Munck, Van Landuyt et al. 2005). This is followed by elution of the degradation products and inadequately cured components into stored water (Hashimoto, Ohno et al. 2002, De Munck, Van Landuyt et al. 2005). Water could also compromise the mechanical properties of the resin-dentin interface by reducing the frictional forces between polymer chains, which is called plasticization (De Munck, Van Landuyt et al. 2005).

Based on the scientific evidence, researchers have standardized the short-term and long-term water storage protocols to be used as laboratory aging techniques with bond strength testing. Short-term testing involves storage of specimens in water for 24hours at 37^0C. Long-term testing involves 6 months of water storage at 37^0C, with the media changed every 7 days to avoid potential microbial overgrowth (ISO/TC 2015).

Thermocycling

Thermocycling is a laboratory aging technique that is simulative of the temperature changes in the oral cavity, and has been used to test the long-term bonding properties of adhesives (Miyazaki, Sato et al. 1998, Nikaido, Kunzelmann et al. 2002, Bedran-de-Castro, Pereira et al. 2004, Mitsui, Peris et al. 2006, Asaka, Amano et al. 2007, Xie, Han et al. 2010, Poptani, Gohil et al. 2012, El-Damanhoury and Gaintantzopoulou 2015). The adhesive joint is subjected to hydrolysis and is stressed by the cyclic expansion and contraction following the hot and cold temperature changes. Consequently, there is a breach in the integrity of the resin-dentin bond and the

surrounding fluid can infiltrate through these gaps at the interface (Gale and Darvell 1999, De Munck, Van Landuyt et al. 2005).

The regimen proposed by the ISO/TS 11450 standard includes 500 cycles in water between 5 – 55^{o}C (ISO/TC 2015). The number of cycles required to produce an adequate aging effect has always been debated. Researchers have advocated against 500 cycles and have instead used up to 100,000 cycles to test bond longevity (Van Meerbeek, Peumans et al. 2010, Morresi, D'Amario et al. 2014). Gale and Darvell (1999) suggested that 10,000 cycles were needed to simulate one year of aging observed clinically. Based on their literature review of the temperatures taken in the oral cavity, they established that extreme temperatures should be avoided and recommended a thermocycling protocol of 35^{o}C (28 s), 15^{o}C (2 s), 35^{o}C (28 s) and 45^{o}C (2 s) (Gale and Darvell 1999). As evident from the discussion, there is lack of unanimity among the researchers regarding the thermocycling regimen and it is difficult to correlate the data from different studies.

Biodegradation

Plaque biofilm is comprised of bacteria, proteins, acids, water and extracellular matrix (Steinberg and Eyal 2002, Heymann, Edward J. Swift et al. 2013). The biofilm formation begins with adsorption of salivary proteins followed by bacterial colonization (Steinberg and Eyal 2002). It is believed that certain environmental factors (e.g. frequent intake of sugars, poor oral hygiene, intraoral appliances) can cause a shift in the dental plaque ecosystem (Marsh 2003) favoring the growth of cariogenic bacteria such as *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus*. These bacteria are acid tolerant and among the strongest acidogens, capable of metabolizing dietary sugars into organic acids and adhesive polysaccharides (Steinberg and Eyal 2002, Marsh 2003, Hahnel, Muhlbauer et al. 2012, Conrads, de Soet et al. 2014). *S. sobrinus* may be

associated with an even higher caries risk than *S. mutans* (Conrads, de Soet et al. 2014) but is not as prevalent as *S. mutans.*

Oral bacteria are capable of forming a biofilm on both hard and soft tissues in the oral cavity, including restorative materials like amalgam, resin composites and ceramics (Busscher, Rinastiti et al. 2010). It has been established that, when compared to other dental materials, more dental plaque accumulates on resin composites than on other restorative materials (Svanberg, Mjor et al. 1990, Beyth, Domb et al. 2007, Beyth, Bahir et al. 2008) and the percentage of viable bacteria is higher (Auschill, Arweiler et al. 2002). Furthermore, lab studies have shown that resin monomers that have leached into the oral cavity as a result of incomplete polymerization or degradation can alter the expression of virulence factors and promote the growth of cariogenic bacteria such as *S. mutans, L. acidophilus and S. sobrinus* (Hansel, Leyhausen et al. 1998, Kawai and Tsuchitani 2000, Khalichi, Cvitkovitch et al. 2004, Khalichi, Singh et al. 2009, Singh, Khalichi et al. 2009, Busscher, Rinastiti et al. 2010). It is clear from this discussion that resin-based dental materials may affect at least some members of the oral microflora, but the extent to which the oral microflora affects resin-based dental materials is uncertain. Different models have been used to test this possibility. While some of the early research used a simple model based on salivary enzymes, more recent studies have used a more complex bacterial challenge.

Human saliva-derived esterases were proven to catalyze the hydrolysis of monomers found in resin composites and adhesives, such as 2,2-bis [4-(2-hydroxy-3-methacryloxypropoxy) phenyl] propane (Bis-GMA) and triethylene glycol dimethacrylate (TEGDMA) at their ester linkages (Santerre, Shajii et al. 1999, Santerre, Shajii et al. 2001, Jaffer, Finer et al. 2002, Finer and Santerre 2003, Finer and Santerre 2004a, Finer and Santerre 2004b, Finer and Santerre 2007). The degradation by-products formed included methacrylic acid (MA), triethylene glycol (TEG) and bis-hydroxy-propoxy-phenyl-propane (Bis-HPPP) (Santerre, Shajii et al. 1999, Jaffer, Finer et al.

2002, Finer and Santerre 2004b). Of these by-products Bis-HPPP was found to be more specific to the degradation of Bis-GMA in the resin matrix (Finer and Santerre 2007). Further, it was demonstrated that the breakdown of marginal resin composite and adhesive by salivary esterases could result in increased penetration of plaque bacteria. These infiltrated bacteria resulted in destruction at the adhesive interface (Kermanshahi, Santerre et al. 2010). Another study proved that the enzymatic hydrolysis following a salivary esterase exposure compromised the fracture toughness and longevity of the dental bonding agent (Shokati, Tam et al. 2010).

S. mutans-based biofilm has been shown to increase surface roughness of resin composites which results in more bacterial deposition and further surface deterioration even though micro-hardness is unaffected (Beyth, Bahir et al. 2008). In addition to the surface properties, the bacterial challenge has been shown to disrupt the integrity of the resin-dentin bond (Bourbia, Ma et al. 2013, Mutluay, Zhang et al. 2013, Borges, Kochhann et al. 2014, Li, Carrera et al. 2014). As a result, lower bond strength (Mutluay, Zhang et al. 2013, Borges, Kochhann et al. 2014, Li, Carrera et al. 2014) fatigue resistance (Mutluay, Zhang et al. 2013) of the adhesive-dentin interface was observed with specimens subjected to bacterial challenge for up to 14 days when compared to 15-90 days of water storage.

Most of the aforementioned studies have used *S. mutans*-based biofilms. *S. mutans* metabolizes carbohydrates, and produces acids and esterases, which have been shown to demineralize tooth tissues (enamel and dentin) and hydrolyze the resin co Thesis Draft mposites and adhesives respectively (Bourbia, Ma et al. 2013, Spencer, Ye et al. 2014). Furthermore, it was shown that strain UA159 had more esterase activity than other *S. mutans* strains that were tested (Bourbia, Ma et al. 2013). Studies have also shown that these acids produced by the plaque bacteria can activate the catalytic activity of matrix metalloproteinase (MMPs) and cysteine cathepsin proteolytic enzymes. These

enzymes contribute to the weakening of adhesive-dentin bonds by hydrolyzing and disrupting the dentin collagen fibers (Delaviz, Finer et al. 2014, Li, Majd et al. 2015).

While the use of *S. mutans*-based single species biofilm is more common, some of the more recent studies have used multi-species biofilm model to mimic the oral environment. In these cases plaque samples were obtained from human volunteers (Borges, Kochhann et al. 2014, Li, Carrera et al. 2014). However, the disadvantage in such a model is that it is difficult to standardize or control overgrowth of any one bacterial species.

Brain Heart Infusion (BHI) broth with (Mutluay, Zhang et al. 2013, Borges, Kochhann et al. 2014, Li, Carrera et al. 2014) and without (Beyth, Bahir et al. 2008, Bourbia, Ma et al. 2013) sucrose has been used for growing the bacteria. The rationale behind using sucrose is to simulate a cariogenic challenge (Borges, Kochhann et al. 2014, Li, Carrera et al. 2014). Some researchers have used nail polish varnish to protect the dentin and composite surfaces while attempting to expose only the adhesive interface to the biofilm challenge (Borges, Kochhann et al. 2014). Nevertheless, this could negatively affect specimen fixation and thereby jeopardize microtensile bond strength testing.

It is evident from these studies that there is a dynamic interaction between the plaque bacteria and the resin materials in the oral cavity. Cariogenic bacteria such as *S. mutans* and *S. sobrinus* produce acids and esterases, which degrade the adhesive-dentin bond and compromise its mechanical properties. Therefore, biofilm challenge can be used as an aging model with laboratory bond strength testing to assess the durability of dental adhesives.

Dental manufacturers are taking advantage of the ambiguities in the materials testing system and are able to release new dental adhesives to market with minimal testing and evidence supporting the material's success. For instance, the American Dental Association (ADA) guidelines for awarding a 'Seal of Acceptance' for dental adhesives and resin-based composites includes laboratory testing and two clinical trials of the

product over 18 months (ADA 1993, ADA 2001a, ADA 2001b, Heintze and Zimmerli 2011). However, in 2008 the program was dropped due to lack of participation from the dental companies (Berthold 2004, Heintze, Thunpithayakul et al. 2011, Heintze and Zimmerli 2011). According to FDA's (U.S. Food and Drug Administration) 510(k) premarket notification, dental bonding agents are classified as class II (medium-risk 510(k)) devices. A new adhesive can be deemed 'substantially similar' to a material marketed before May 28, 1976, thus allowing the manufacturer to start selling without additional testing and clinical trials needed via pre-market approval, PMA (fda.gov). Therefore, due to the lack of regulatory requirement or consumer demand, very few clinical trials are conducted and when conducted are post-market akin to the Phase IV clinical trials for drugs and higher-risk medical devices.

These gaps reinforce the need for a laboratory aging method that is short-term and can aid in predicting the long-term performance of dental adhesives. A biofilm model is simulative of the oral environment and will help in expediting the aging process of adhesive-dentin bonds. Therefore, the aim of the present study is to adopt a practical biofilm model and work towards standardizing it for lab testing of newer adhesives. The study will compare the degradative effect of 15 days exposure to a *Streptococcus mutans* or *Streptococcus sobrinus* cariogenic bacteria-based biofilm on the microtensile bond strengths of dental adhesive with that obtained using the ISO recommendation of 6 months of water storage (ISO/TC 2015).

CHAPTER III: MATERIALS AND METHODS

Overview

The objective of the present study was to develop a biofilm-based aging/storage model for lab testing of newer dental adhesives. To accomplish this we tested if a 15-day biofilm challenge with cariogenic bacterial species, *Streptococcus mutans* (SM) or *Streptococcus sobrinus* (SS) would produce a similar or greater reduction in microtensile bond strength (μTBS) of dental adhesives as 6 months of water storage (WS).

Research Questions

Will the exposure to 15 days of *Streptococcus mutans* (SM)-based biofilm cause a similar or more significant reduction in microtensile bond strengths (MPa) of dental adhesives when compared to exposure to 6 months water storage (WS)?

Will the exposure to 15 days of *Streptococcus mutans* (SM)-based biofilm result in more interfacial failures of dental adhesives when compared to exposure to 6 months water storage (WS)?

Will the exposure to *Streptococcus sobrinus* (SS)-based biofilm represent a greater bacterial challenge than exposure to *Streptococcus mutans* (SM)-based biofilm?

Null Hypotheses

$H_{o}1$: There is no difference in the microtensile bond strength (μTBS) values of resin-dentin bonds aged using *Streptococcus mutans* (SM)-based biofilm for 15 days when compared to the values obtained with resin-dentin bonds aged using 6 months of water storage (WS)

$H_{o}2$: There is no difference in the μTBS values of resin-dentin bonds aged in water for 6 months when compared to the values obtained with resin-dentin bonds aged in water for 5.5 months followed by 15 days of SM-based biofilm storage

H_o3: There is no difference in the μTBS values of resin-dentin bonds after 15 days of aging using SM-based biofilm when compared to the values obtained with resin-dentin bonds aged for 15 days using a SS-based biofilm

H_o4: There is no difference in the failure mode observed with resin-dentin bonds aged using SM-based biofilm for 15 days when compared to that observed with resin-dentin bonds aged using 6 months of water storage

H_o5: There is no difference in the failure mode observed with resin-dentin bonds aged in water for 6 months when compared to that observed with resin-dentin bonds aged in water for 5.5 months followed by 15 days of SM storage

H_o6: There is no difference in the failure mode observed with resin-dentin bonds aged for 15 days using a SM-based biofilm when compared to that observed with resin-dentin bonds aged for 15 days using a SS-based biofilm

Variables

The independent variables of the study were the four storage conditions that were tested. The dependent variables were the microtensile bond strengths (MPa) and the fracture location as observed using light microscopy.

Study Design Outline

The following section is a flowchart representation of the research protocol for the pilot and the main experimental study. Specimen preparation, storage methods, microtensile bond strength testing, fractography and statistical analysis are described later in the chapter.

Pilot study design

A 14-day pilot study was conducted to study the effect of four different storage conditions shown below on the μTBS of dental adhesive. The pilot study data was also used for estimating the sample size required for the main experimental study. The flow chart in figure 3 provides an overview of the various steps followed during the pilot study.

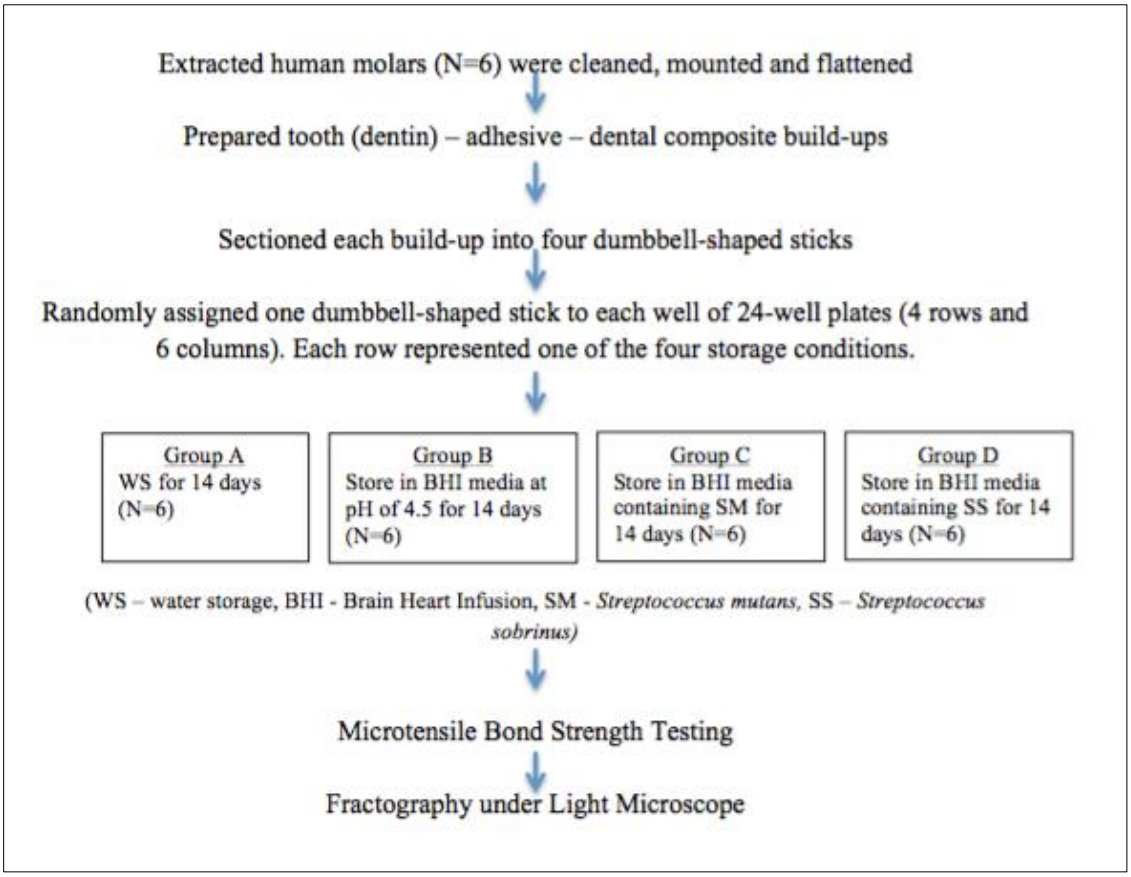

Figure 3: Flowchart of the pilot study design

Main experimental study design

The main experimental study was conducted to study the effect of four different storage conditions shown below on the μTBS of dental adhesive. The flow chart in figure 4 provides an overview of the various steps followed during the study. The detailed methodology is described subsequently.

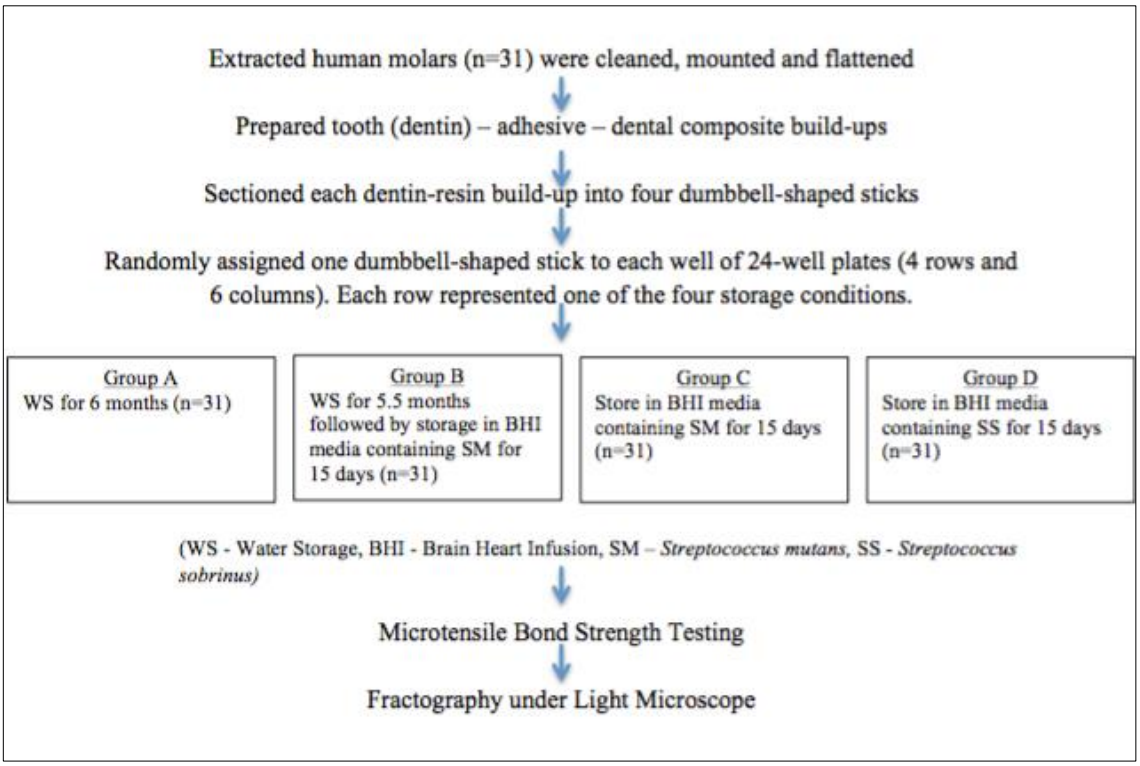

Figure 4: Flowchart of the main experimental study design

Specimen Preparation

Standard guidelines recommended by the ISO/TS 11405 for μTBS testing, such as storage condition, substrate selection and specimen preparation, were followed during the present study (ISO/TC 2015). Non-carious, non-restored extracted human molars were

selected from the pool of extracted teeth at the University of Iowa College of Dentistry and Dental Clinics. The teeth were used within 6 months of extraction but the age of the patient to whom the extracted teeth belong is unknown. These teeth meet institutional review board guidelines as extractions were performed solely for clinical reasons and are untraceable to the patient from which they came. The surface of the teeth was cleaned of gross debris and stored in 0.5% Chloramine-T trihydrate bacteriostatic solution (0.5% of chloramine-T trihydrate mixed with distilled water) at 4°C until they were needed for mounting. The steps of specimen preparation for μTBS testing are illustrated in figure 5.

Using a custom-fabricated mounting device each tooth was embedded in dental stone by their roots with the long axis of each tooth parallel to the walls of the mold. After 10 minutes the mounting device containing the embedded teeth was placed in water at room temperature until the stone was fully set. The occlusal enamel of each tooth was partially removed perpendicular to the long axis of the tooth using a model trimmer with water coolant (12" Super Abrasive Blue Wonder Diamond Wheel, Whip Mix Corporation, Louisville, KY, USA). All teeth were then flattened using a carbide bur (# 55, Brasseler, Savannah, GA, USA) mounted in the Computer Numeric Controlled Specimen Former (University of Iowa, Iowa City, IA, USA) to expose coronal dentin. This was followed by application of etchant, primer and adhesive to the flat dentin surface (figure 5). Optibond FL (Kerr, Orange, CA, USA) 3-step etch-and-rinse adhesive system was used for all the specimens.

According to the manufacturer's instructions, the dentin surface was etched with 37% phosphoric acid for 15 seconds and then rinsed with iodine-disinfected clinic water supply for 15 seconds. The etched surface was blotted with low-lint wipes (Kimwipes, Kimberly-Clark Professional, Roswell, GA, USA) to maintain a moist surface for priming. The Optibond FL primer (Lot Number 5450880) was applied with a microbrush and lightly scrubbed with the same microbrush for 30 seconds followed by air-drying to evaporate all the solvents and achieve a shiny evenly primed surface. Initially the primer

was air-dried gently from a distance and perpendicular to the dentin surface. Gradually, the air syringe was brought closer and dried until there was no further visible fluid movement when using moderate air pressure.

Then Optibond FL adhesive (Lot Number 5461157) was applied using microbrush and light cured for 30 seconds using Optilux 500 light curing unit (Kerr, USA). Immediately after adhesive application, build-ups were constructed with Z-100 (shade A1, Lot Number N578117, expiration-2016, 3M ESPE, St. Paul, MN, USA) hybrid resin-based composite in three increments, the initial increment being of 1 mm followed by 2 increments of 2mm each (figure 5). Each increment was cured for 40 seconds using the same Optilux 500 light-curing unit (Kerr). The tip of the curing light was held ≤ 1mm from the composite surface without touching it. The Optilux 500 light curing unit used in the present study was tested with a MARC^{TM}RC (Managing Accurate Resin Curing -Resin Calibrator) measurement system. The measured excitant irradiance on the MARC^{TM}RC was 1390 mW/cm^2 and the curing unit's irradiance was stable throughout the study as monitored with a radiometer Demetron (Model Number: VCL 500, KERR, Danbury, CT, USA). As per the manufacturer's directions for usage, Optibond FL and Z-100 require a radiant exposure of 18J/cm^2 and 8J/cm^2, respectively. The clinically-relevant light curing unit delivery times of 30 and 40 seconds delivered radiant exposures (41.7 and 55.6 J/cm^2) that assure that the materials used were fully polymerized for this durability study.

After the dental composite build-ups, four 2 mm x 2 mm resin-dentin sticks were formed per tooth using water-cooled triple diamond saw blades mounted in a sectioning machine (Isomet 1000, Buehler, Lake Bluff, IL, USA). Each stick was trimmed into a dumbbell-shaped test specimen (figure 5) producing a round cross-sectional area of 0.5 mm^2, a gauge length of 1 mm, and a radius of curvature or 'neck' of 0.6 mm using a diamond bur (Brasseler, Savannah, GA, USA) mounted in the Specimen Former machine (Computer Numeric Controlled Specimen Former, University of Iowa, Iowa City, IA,

USA).

The dumbbell-shaped specimens were stored in 0.5% Chloramine-T disinfectant solution (0.5% of chloramine-T trihydrate mixed with autoclaved water) for 24 hours. After 24 hours, they were rinsed five times using autoclaved water. The four dumbbell-shaped specimens from each tooth were then randomly assigned to four different storage conditions.

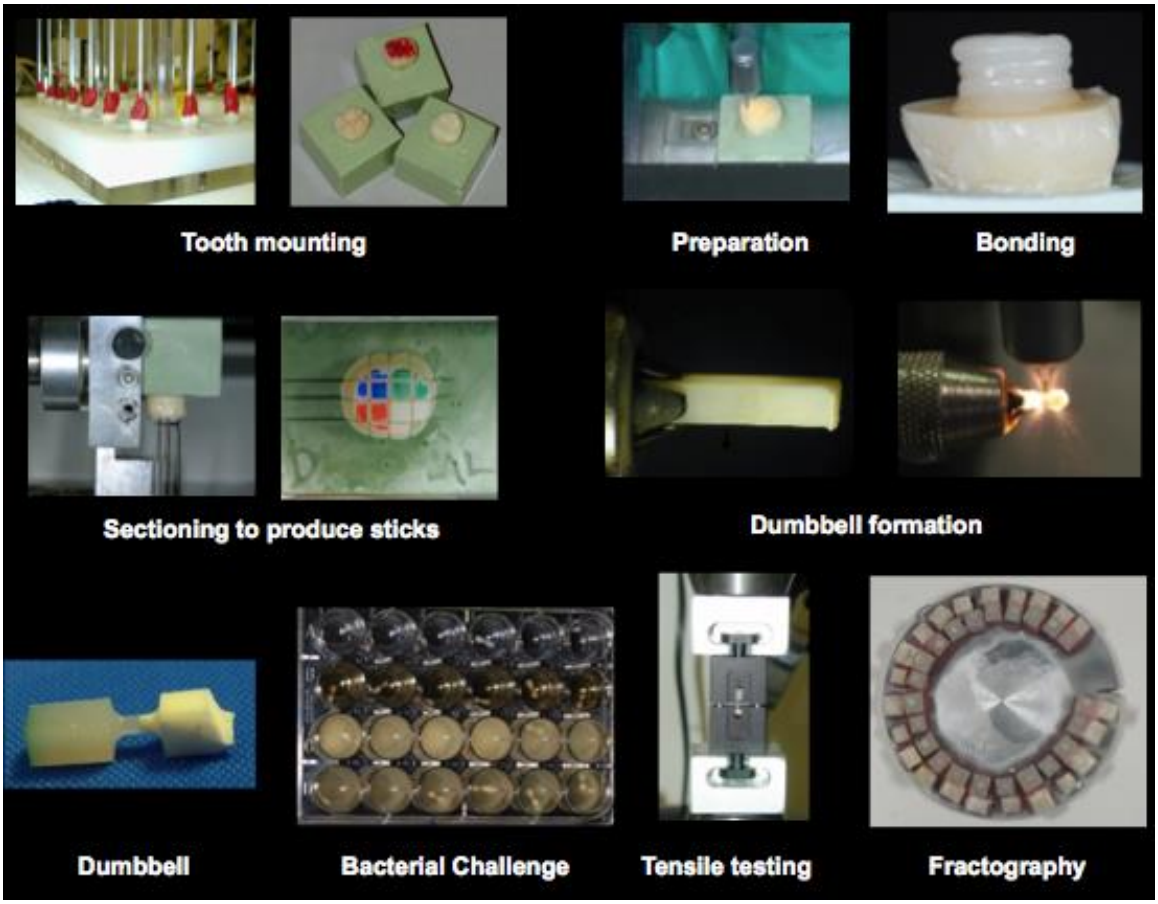

Figure 5: Diagram showing specimen preparation steps for microtensile bond strength testing

Aging/Storage Conditions

The four dumbbell-shaped sticks, obtained from one resin-dentin tooth specimen were randomly placed in a well of a 24-well plate (4 rows and 6 columns) in both the pilot and the main experimental study. The wells were inoculated with 1 mL of the aging solution. Each row of the well plate represented one of the four storage conditions being evaluated (figure 6). The storage conditions for both the pilot study and the main experimental study are described below.

Figure 6: Pilot study aging solutions and the dumbbell-shaped specimens in a 24-well plate

Pilot study

Group A: Storage of specimens for 14 days in autoclaved water at 37^0C. Water was changed every 7 days.

Group B: Storage of specimens for 14 days in Brain Heart Infusion (BHI) medium containing lactic acid at pH 4.5, incubated at 37^0C. The medium was changed every 7 days.

Group C: Storage of specimens in *Streptococcus mutans* (SM)-based biofilm for 14 days, incubated at 37^0C. The growth medium* of the bacterial culture was changed every day for 14 days.

Group D: Storage of specimens in *Streptococcus sobrinus* (SS)-based biofilm for 14 days, incubated at 37^0C. The growth medium* of the bacterial culture was changed every day for 14 days.

Main experimental study

Group A: Storage of specimens for 6 months in autoclaved water at 37^0C. Water was changed every 7 days.

Group B: Storage of specimens for 5.5 months in autoclaved water at 37^0C followed by 15 days of SM storage, incubated at 37^0C. The autoclaved water was changed every 7 days and later the growth medium* of the *Streptococcus mutans* (SM) culture was changed every day for 15 days.

Group C: Storage of specimens in *Streptococcus mutans* (SM)-based biofilm for 15 days, incubated at 37^0C. The growth medium* of the bacterial culture was changed every day for 15 days.

Group D: Storage of specimens in *Streptococcus sobrinus* (SS)-based biofilm for 15 days, incubated at 37^0C. The growth medium* of the bacterial culture was changed every day for 15 days.

A separate set of specimens (n = 4) stored in autoclaved water at 37^0C for 24 hours acted as baseline laboratory control as this adhesive has been used for more than two decades in our laboratory.

Growth Medium Formulation: Streptococcus mutans* (ATCC, UA159, American Type Culture Collection) and *Streptococcus sobrinus* (ATCC 33478) were cultured on Trypticase Soy Agar (TSA) with 5% sheep's blood for 24 hours. Colonies from the blood agar plates were inoculated into Brain Heart Infusion (BHI) broth using a sterile Q-tip. The BHI medium was supplemented with 0.5% sucrose to promote the proliferation of the bacteria and development of a biofilm (Mutluay, Zhang et al. 2013). After the initial 24 hours, daily changes in growth medium consisted of BHI medium without the sucrose to avoid overwhelming growth of bacteria and extremely acidic pH.

Microtensile Bond Strength Testing

Microtensile bond strength testing was performed at room temperature and humidity immediately after removal from storage media (ISO/TC 2015). The test specimens were gripped centrally with respect to the test axis with a non-gluing passive gripping device (Dircks Device, University of Iowa, Iowa city, IA, USA). Microtensile testing was performed at a crosshead speed of 1 mm/min using a calibrated Zwick Material Testing Machine. Bond strengths were expressed in MPa and computed as tensile or pulling force (measured in Newtons per unit area) required to break the bonded assembly. Only those specimens that fractured within the gauge area were included in data analysis.

Fractography under Light Microscopy

The two fractured segments of each specimen were then observed under the light microscope to identify the failure mode. The failure mode at the resin-dentin interface

was classified as adhesive failure if the fracture location was within the adhesive joint. If the fracture was in dentin or within the resin-based composite, the mode was recorded as cohesive or substrate failure. Finally, fractures travelling through the joint into either dentin or resin-based composite were recorded as mixed failures.

Debond Specimen Storage and Electron Microscopy Preparation

One to seven days after testing, the broken halves were placed on aluminum SEM (Scanning Electron Microscope) stubs using flowable composite. For specimen storage, the stubs with the mounted specimens were then placed in 3% glutaraldehyde/3% formaldehyde in sodium cacodylate buffer at pH 7.3 (Tousimis Research Corp., Rockville, MD, USA) overnight at 4^{O}C. Next the samples were removed from the fixative and placed in 0.2 M sodium cacodylate buffer at pH 7.3 for 10 min with two changes of fresh buffer, then dehydrated in ascending grades of ethanol (30, 50, 70%) for 10 min with two changes and air-dried. The specimens were then held at 70% ethanol for long-term storage. These specimens can later be further dehydrated (90% and 100%) and sputter-coated with gold for SEM examination as needed (Armstrong, Keller et al. 2001b).

Statistical Analysis

The data of the 14-day pilot study was analyzed using one-way ANOVA with repeated measures and post-hoc contrasts. Additionally, a power analysis was conducted (using nQuery + nTerim 2.0 software) based on the same pilot study data that guided sample size selection for the main experimental study.

Two sets of statistical analysis were done for main experimental study data, first one considering independence of specimens and a second one taking into account the clustering of specimens (four specimens from the same tooth). When considering

independence of specimens, one-way ANOVA with post-hoc Tukey's HSD (Honestly Significant Difference) test and parametric Weibull regression model with Wald chi-square test were used to determine the effect of four different storage conditions on μTBS (MPa) of dental adhesive. When tooth dependency (four specimens from the same tooth) was taken into account, simple random effect in the Mixed Model ANOVA (i.e. to allow correlation between four specimens from the same tooth) with tests for differences of least squares means and the random effect Weibull regression model with nonparametric Wilcoxon signed-rank test were applied to evaluate the effect of the type of storage condition on μTBS.

Association between failure modes and the type of storage condition were analyzed using a chi-square test. All tests utilized a significance level of 0.05. Data analyses were performed using the statistical package SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA).

CHAPTER IV: RESULTS

Overview

The present study tested if a 15-day biofilm challenge with cariogenic bacterial species, *Streptococcus mutans* (SM) or *Streptococcus sobrinus* (SS) would produce similar or greater reduction in microtensile bond strength (μTBS) of dental adhesives as 6 months of water storage (WS). The results of the pilot study and the main experimental study are discussed in this chapter. In addition, the first section elaborates on an experiment that was done to assess the difference between the acidogenicity of *Streptococcus sobrinus* (SS) and *Streptococcus mutans* (SM).

Preliminary Bacterial Acidogenic Challenges

To rule out pH as an experimental variable, *Streptococcus sobrinus* (SS) and *Streptococcus mutans* (SM) were incubated in BHI (Brain Heart Infusion) media and the pH decrease was measured over 18 hours. *S. sobrinus* and *S. mutans* were cultured in 75 ml of growth medium in a flask. The baseline pH was recorded as 7.4. After 48 hours the BHI medium was changed in both flasks and the drop in pH was recorded every hour for 6 hours with a final reading taken at 18 hours (Table 1). From the results in Table 1, it is evident that a similar trend in the drop of pH is seen in both *S. sobrinus*- and *S. mutans*-incubated BHI media. These findings support the assumption that pH differences between *S. sobrinus* and *S. mutans* is unlikely to be a variable affecting the outcome of the study.

Table 1: pH recorded after incubation of *Streptococcus mutans* (SM) and *Streptococcus sobrinus* (SS) in BHI media

Time Hour(s)	pH	
	Streptococcus mutans (SM)	*Streptococcus sobrinus* (SS)
0	6.78	6.15
1	6.23	6.05
2	5.84	5.95
3	5.17	5.08
4	4.84	4.74
5	4.65	4.57
6	4.49	4.45
18 hours	3.97	3.92

Pilot Study Results

As part of a 14-day pilot study, the effect of four different storage conditions (14-day WS, 14-day acidic BHI medium, 14-day SM, and 14-day SS) on microtensile bond strength of dental adhesive was evaluated. The data were analyzed using one-way ANOVA with repeated measures and post-hoc contrasts. Additionally, a power analysis was conducted (using nQuery + nTerim 2.0 software) based on the same pilot study data that guided sample size selection for the main experimental study.

One-way ANOVA with repeated measures revealed that there was a significant effect ($p=0.017$) for the type of storage condition on microtensile bond strength of dental adhesive (Table 2). The post-hoc contrasts showed that there were no statistically significant differences between the microtensile bond strengths (μTBS) of 14-day WS

(38.83 ± 11.40MPa), 14-day SM (38.35 ± 12.00MPa), and 14-day SS (24.37 ± 11.46MPa) (p=0.1117). However, it was observed that the μTBS of 14-day acidic BHI medium (5.01 ± 3.19MPa) was significantly lower than the μTBS values observed in the other three storage groups (Table 2). From the physical appearance, it seemed like the acidic medium produced excessive demineralization in the specimens, resulting in dark and very soft dentin substrate. This observation was further confirmed by the fractographic analysis under a light microscope, which revealed that 50% of the specimens in the 14-day acidic BHI medium group failed cohesively within the dentin substrate whereas the specimens of the other groups majorly failed at the adhesive joint (Table 2).

As a result of the sizeable discrepancy in μTBS between samples, an additional analysis was conducted using the one-way ANOVA with repeated measures excluding the 14-day acidic BHI medium group. This analysis revealed that with the current sample size of 6 per group, no significant differences (p=0.1117) were found between the microtensile bond strengths of the remaining three groups (14-day WS, 14-day SM, and 14-day SS). Nonetheless, the p-value was suggestive of an effect that might be shown statistically in an experiment with a larger sample size. A power analysis was conducted for these three groups. The analysis indicated that 11 - 12 teeth per group would be required for the study design, which would have the capability to distinguish a mean difference of approximately 14.22 MPa with 80% or higher power using the one-way repeated measures ANOVA.

In addition to ANOVA, we also used a Weibull regression model for data analysis of the main experimental study. The study design involves clustering of specimens, which is, obtaining multiple specimens (four dumbbells in this case) from the same tooth. This dependency of the specimens on a single extracted tooth might affect the outcome (microtensile bond strength in the present study) and therefore cannot be ignored as a factor when analyzing the raw data. Since the Weibull regression model takes clustering

of specimens into consideration, it was determined to be an appropriate statistical analysis for the present study (Hougaard 1995, Sahu, Dey et al. 1997, Armstrong, Keller et al. 2001b). For applying the Weibull regression model, at least 25 - 30 samples per group are required (McCabe and Walls 1986). Therefore, 30 samples per group were chosen to be the sample size for the main experimental study. Also, due to the extremely low μTBS and excessive dentin demineralization, the acidic BHI medium group was replaced with a 5.5-month WS +15-day SM group.

Table 2: Pilot study: microtensile bond strengths and failure modes of four types of storage conditions

Groups (N=6)	Description of Aging Conditions	Microtensile bond strength Mean ± SD (MPa) *	Failure Modes N (%)	
			Adhesive (N=18)	Cohesive (N=6)
Group A	14 days WS	38.83 ± 11.40^{A}	5 (83.3)	1 (16.7)
Group B	14 days BHI medium at pH 4.5	5.01 ± 3.19^{B}	3 (50)	3 (50)
Group C	14 days SM storage	38.35 ± 12.00^{A}	4 (66.7)	2 (33.3)
Group D	14 days SS storage	24.37 ± 11.46^{A}	6 (100)	0 (0)

*Column means with dissimilar letters are statistically significant using the post-hoc contrasts ($p>0.05$).
WS – water storage, BHI – Brain Heart Infusion, SS - *Streptococcus sobrinus*, SM - *Streptococcus mutans*, MPa – megapascal, SD – standard deviation

Main Experimental Study Results

The main experimental study was conducted to evaluate the effect of four different storage conditions (6 months water storage, 6 months water storage + 15 days SM storage, 15 days SM storage, and 15 days SS storage) on microtensile bond strength of dental adhesive. Because of the reasons discussed above, two sets of statistical analysis

were done, the first considering the independence of the specimens and the second taking into account the clustering of specimens. When considering the independence of specimens, a one-way ANOVA with post-hoc Tukey's HSD (Honestly Significant Difference) test and parametric Weibull regression model with Wald chi-square test were used to determine the effect of four different storage conditions on μTBS (MPa) of dental adhesive. When tooth dependency (four specimens from the same tooth) was taken into account, simple random effect in the Mixed Model ANOVA (i.e. to allow correlation between four specimens from the same tooth) with tests for differences of least squares means and the random effect Weibull regression model with nonparametric Wilcoxon signed-rank test were applied to evaluate the effect of the type of storage condition on μTBS. An association between failure modes and the type of storage condition were analyzed using a chi-square test. All tests utilized a significance level of 0.05. Data analyses were performed using the statistical package SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA).

Considering independence of the specimens

Results of the one-way ANOVA (Table 3) revealed that there was a significant effect of the type of storage condition on the μTBS ($p<0.0001$) of the adhesive tested. The post-hoc Tukey's HSD test indicated that the mean μTBS observed in 6-month WS (49.69 ± 15.53Mpa) was significantly greater than the values observed in 5.5-month WS and 15-day SM (19.26 ± 6.26Mpa), 15-day SM (19.92 ± 5.86Mpa) and 15-day SS (23.58 ± 7.88MPa), while no significant differences were found between the other storage conditions, 5.5-month WS with 15-day SM, 15-day SM, and 15-day SS (Table 3).

The parametric Weibull regression model corroborated the one-way ANOVA in that there was a significant relationship between the type of storage condition and the μTBS ($p<0.0001$). The Wald chi-square test showed same statistical results as post-hoc

Tukey's HSD test with one exception, in that the median μTBS observed in 15-day SS was significantly greater than those observed in 5.5- month WS with 15-day SM and 15-day SM (Table 4). Table 4 reports Weibull parameter estimates at 95% confidence intervals. Figure 7 displays the Weibull plot of probability of failure (%) against the μTBS at failure (MPa) for each type of storage condition.

Table 3: Microtensile bond strengths of four types of storage conditions considering independence of the specimens

Groups (N=31)	Description of Aging Conditions	Microtensile bond strength Mean ± SD (MPa) *
Group A	6 months WS	49.69 ± 15.53^{A}
Group B	5.5 months WS + 15 days SM storage	19.26 ± 6.26^{B}
Group C	15 days SM storage	19.92 ± 5.86^{B}
Group D	15 days SS storage	23.58 ± 7.88^{B}

*Column means with dissimilar letters are statistically significant using post-hoc Tukey's HSD tests ($p>0.05$).
WS – water storage, SS - *Streptococcus sobrinus*, SM - *Streptococcus mutans*, MPa – megapascal, SD – standard deviation
.

Table 4: Weibull parameters for the microtensile bond strength measurements of four types of storage conditions considering independence of the specimens

Storage Conditions	N	Median** (stderr) (MPa)	95 % Confidence Interval	Scale (stderr)	95 % Confidence Interval	Shape (stderr)	95 % Confidence Interval	5% chance of Failure (stderr)	95 % Confidence Interval	95% chance of Failure (MPa) (stderr)	95 % Confidence Interval)
6 mo WS	31	48.60 (2.86) ^{A}	43.30-54.54	54.01 (2.87)	48.68-59.94	3.47 (0.54)	2.55-4.71	22.93 (3.54)	16.94-31.05	74.12 (4.76)	65.35-84.08
5.5 mo WS + 15 d SM	31	19.22 (1.14) ^{B}	17.12-21.59	21.32 (1.13)	19.22-23.66	3.54 (0.52)	2.65-4.72	9.20 (1.36)	6.89-12.30	29.08 (1.72)	25.90-32.66
15 d SM	31	20.02 (1.11) ^{B}	17.95-22.32	22.05 (1.10)	19.99-24.31	3.79 (0.52)	2.90-4.96	10.08 (1.33)	7.78-13.05	29.44 (1.56)	26.54-32.65
15 d SS	31	23.54 (1.48) ^{C}	20.81-26.63	26.26 (1.48)	23.51-29.34	3.35 (0.47)	2.54-4.41	10.82 (1.64)	8.03-14.56	36.45 (2.21)	32.36-41.05

**Column median with dissimilar letters are statistically significant using the Wald chi-square test
WS – water storage, mo – months, d – days, SS - *Streptococcus sobrinus*, SM - *Streptococcus mutans*, MPa – megapascal

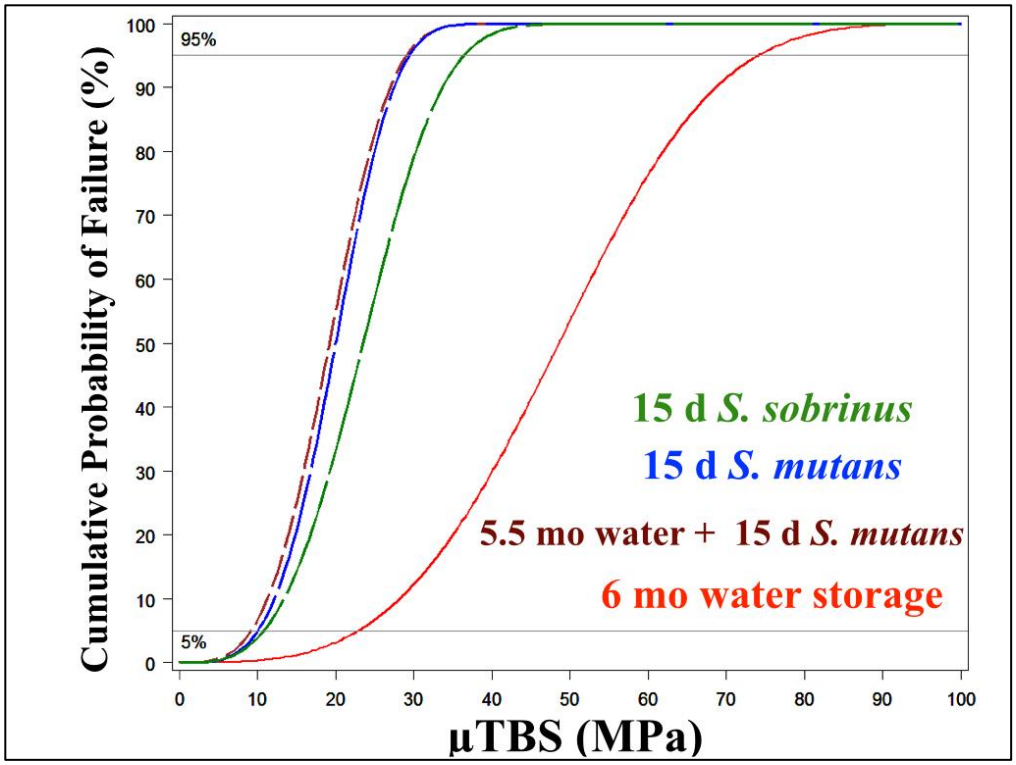

Figure 7: Weibull plot of probability of failure (%) against the microtensile bond strength to failure (MPa) for each of the storage conditions

Considering tooth dependency

Both simple random effect in the Mixed Model ANOVA (i.e. to allow correlated measurements obtained from the same tooth) and the parametric Weibull regression model with random effect (Table 5) revealed that there was a statistically significant effect of the type of storage condition on the µTBS ($p<0.0001$).

The tests for differences of least squares means was used to compare the mean microtensile bond strengths and the nonparametric Wilcoxon signed-rank test was used to compare the median microtensile bond strengths of the four storage conditions, respectively. Both the tests indicated that the µTBS observed in 6-month water storage was significantly greater than that observed in the other three types of storage conditions.

Further, the μTBS observed in 15-day SS was significantly greater than that observed in 5.5-month water with 15-day SM and 15-day SM. However, no significant difference was found between the latter two groups (Table 5).

Differences in statistical significance were observed between the results of ANOVA and a Weibull regression analysis when tooth dependency was taken into consideration. This indicates the importance of applying the Weibull model in clustered specimens like the present situation.

Table 5: Microtensile bond strengths of four types of storage conditions considering tooth dependency

Groups (N=31)	Description of Aging Conditions	Mean (SD)/Median (SD) Microtensile Bond Strength (MPa) **
Group A	6 months WS	49.69 (15.53)/48.6 (2.86) ^{A}
Group B	5.5 months WS + 15 days SM storage	19.26 (6.26)/19.22 (1.14) ^{B}
Group C	15 days SM storage	19.92 (5.86)/20.02 (1.11) ^{B}
Group D	15 days SS storage	23.58 (7.88)/23.54 (1.48) ^{C}

**Column means /medians with dissimilar letters are statistically significant using mixed model ANOVA with tests for differences of least squares means and Weibull regression model with nonparametric Wilcoxon signed-rank test, respectively
WS – water storage, SS - *Streptococcus sobrinus*, SM - *Streptococcus mutans*, MPa – megapascal, SD – standard deviation

Failure Modes and Types of Storage Conditions

A chi-square test showed that there was a significant association (p=0.0463) between failure mode and the type of storage condition (Table 6). Of the 124 specimens, 26.6% (n=33) had an adhesive failure mode and 73.4% (n=91) had a cohesive failure

mode in dentin. Specimens observed in 5.5-month WS with 15-day SM (74.2%), 15-day SM (83.9%) and 15-day SS (80.6%) were more likely to have cohesive failures in dentin than that observed in 6-month WS (54.8%).

Table 6: Comparison of failure modes with the types of storage conditions

Failure Modes	Types of Storage Conditions				p-value
	6 months WS n (%)	5.5 months WS + 15 days SM storage n (%)	15 Days SM n (%)	15 Days SS n (%)	
Adhesive (n=33)	14 (45.2)	8 (25.8)	5 (16.1)	6 (19.4)	0.0463
Cohesive (n=91)	17 (54.8)	23 (74.2)	26 (83.9)	25 (80.6)	

(WS – water storage, SS - *Streptococcus sobrinus*, SM - *Streptococcus mutans*)

Statement of Hypotheses

Based on the results of microtensile bond strengths and failure modes for each of the storage conditions, the following null hypotheses were either accepted or rejected (Table 7).

Table 7. Experimental study hypotheses

Null Hypothesis tested	Conclusion
$H_{o}1$: There is no difference in the microtensile bond strength (μTBS) values of resin-dentin bonds aged using *Streptococcus mutans* (SM)-based biofilm for 15 days when compared to the values obtained with resin-dentin bonds aged using 6 months of water storage (WS)	Rejected
$H_{o}2$: There is no difference in the μTBS values of resin-dentin bonds aged in water for 6 months when compared to the values obtained with resin-dentin bonds aged in water for 5.5 months followed by 15 days of SM-based biofilm storage	Rejected
$H_{o}3$: There is no difference in the μTBS values of resin-dentin bonds after 15 days of aging using SM-based biofilm when compared to the values obtained with resin-dentin bonds aged for 15 days using a *Streptococcus sobrinus* (SS)-based biofilm	Rejected
$H_{o}4$: There is no difference in the failure mode observed with resin-dentin bonds aged using SM-based biofilm for 15 days when compared to that observed with resin-dentin bonds aged using 6 months of water storage	Rejected
$H_{o}5$: There is no difference in the failure mode observed with resin-dentin bonds aged in water for 6 months when compared to that observed with resin-dentin bonds aged in water for 5.5 months followed by 15 days of SM storage	Rejected
$H_{o}6$: There is no difference in the failure mode observed with resin-dentin bonds aged for 15 days using a SM-based biofilm when compared to that observed with resin-dentin bonds aged for 15 days using a SS-based biofilm	Fail to Reject

CHAPTER V: DISCUSSION

Overview

Water storage and thermocycling are common *in vitro* aging methods used to challenge the mechanical properties and hydrolytic stability of dental adhesives when evaluating their bonding efficacy. The present study was done to assess if a biofilm challenge can be used as a method of accelerated aging for laboratory microtensile bond strength testing of dental adhesives. This type of aging model would additionally test biochemical and enzymatic stability, which are equally important for the durability of bonding agents. We compared the effect of two weeks exposure to *Streptococcus mutans* or *Streptococcus sobrinus* cariogenic bacteria-based biofilm and 6 months of water storage on microtensile bond strength (μTBS) of dental adhesive. There have been a few studies that evaluated the effect of biofilm degradation on bond strength of dental adhesives (Mutluay, Zhang et al. 2013, Borges, Kochhann et al. 2014, De Carvalho, Puppin-Rontani et al. 2014, Li, Carrera et al. 2014). But none of them have compared the effect of biodegradation to the ISO recommendation of 6 months water storage aging before microtensile bond strength testing of resin adhesion to tooth structure (ISO/TC 2015).

Microtensile Bond Strength Test

Results of this study revealed that the type of storage condition elicited a significant effect on the μTBS ($p<0.0001$) of the adhesive tested. The first null hypothesis $H_{o}1$ tested was that cariogenic bacterial species, *Streptococcus mutans* and *Streptococcus sobrinus* would produce similar reduction in μTBS of dental adhesives as water storage. It was rejected because 15 days of *S. mutans*-based or *S. sobrinus*-based biofilm challenge produced more degradation of the resin-dentin interface, resulting in significantly lower μTBS values (19.92±5.86MPa and 23.58±7.88MPa, respectively),

than those obtained with specimens exposed to 6 months of water storage (49.69±15.53MPa). Also, significantly lower μTBS values were observed in specimens stored in water for 5.5 months followed by 15 days of *S. mutans* challenge (19.26±6MPa) when compared to those stored in water for 6 months (49.69±15.53MPa). Thus, the second null hypothesis $H_{o}2$ was also rejected. These results are in agreement with a previous study done by Mutlay et al. (2013) to evaluate the mechanical properties of dental adhesives following a biofilm challenge (Mutluay, Zhang et al. 2013). That study found that 14 days of *S. mutans*-based biofilm exposure caused significant reduction in the flexural strength and fatigue resistance of resin-dentin interfaces whereas 14 days of water storage resulted in no significant reduction of μTBS (Mutluay, Zhang et al. 2013). Dumbbell-shaped resin-dentin specimens were used in the present study whereas rectangular beams with twin bonded resin-dentin interfaces were used in the Mutlay et al. (2013) study. In contrast, Borges et al. (2014) did a similar study and observed no significant differences in μTBS of dental adhesive exposed to 10 days of mixed-species biofilm compared to 3 months of water storage (Borges, Kochhann et al. 2014). A possible explanation is the saliva-like medium containing hydroxyapatite that was used for the growth of bacteria. As mentioned by the authors, this medium could have lead to remineralization, thereby reversing the degradative effect of the biofilm. Also, these resin-dentin specimens were exposed to only 10 days of biofilm challenge as opposed to 15 days in the present study (Borges, Kochhann et al. 2014). They used rectangular resin-dentin beams as opposed to the dumbbell-shaped specimens used in the present study. The advantage of using dumbbell-shaped specimens, as in the current study, is that there is more uniform distribution of stress under tensile load (Soares, Soares et al. 2008). Like some other studies related to biodegradation done in the past (Zhang, Cheng et al. 2013, Li, Carrera et al. 2014), Borges et al. used a multi-species biofilm, which was cultured from saliva of human volunteers (Borges, Kochhann et al. 2014). Although multi-species biofilms are more clinically simulative, the disadvantage is that it is difficult to

standardize and control any one bacterial species from overgrowing the others. Additionally, our objective was to identify a practical approach to test the biostability of adhesive resin bonding to dentin that could be widely adopted by the research and standardization community. Therefore, *S. mutans* - and *S. sobrinus* - based single species biofilm was used in the present study.

Aging Conditions

S. mutans and *S. sobrinus* may be part of dental plaque biofilms and are consistently correlated with dental caries in humans (Loesche 1986, Okada, Soda et al. 2005, Choi, Lee et al. 2009, Hahnel, Muhlbauer et al. 2012). Factors responsible for their pathogenicity and survivability include sucrose-enhanced adhesion, broad fermentative capabilities that produce organic acids, acid tolerance and the synthesis of degradative enzymes (Steinberg and Eyal 2002, Conrads, de Soet et al. 2014). The acids and esterase enzyme of *S. mutans* have been proven to produce dentin demineralization and breakdown of the ester bonds of resin monomers respectively (Bourbia, Ma et al. 2013, Borges, Kochhann et al. 2014, Spencer, Ye et al. 2014). Also, *S. mutans* exhibits affinity towards certain resin monomers resulting in greater degradation of materials in which they are incorporated (Bourbia, Ma et al. 2013). *S. sobrinus* also displays differing affinities towards various dental materials (Steinberg and Eyal 2002, Hahnel, Muhlbauer et al. 2012). However, little is known about the degradative effects of sobrinus streptococci on resin-based dental materials specifically. Strain UA159 was selected over the other strains of *S. mutans* because it is a well-studied strain whose genomic sequence has been determined (Ajdic, McShan et al. 2002). Also, among strains of *S. mutans* that have been tested, UA159 was shown to have the highest esterase activity against resin substrate commonly present in dental adhesives and composites (Bourbia, Ma et al.

2013). *S. sobrinus* strain ATCC 33478 was utilized because this strain is a type strain displaying the typical properties of the species.

S. sobrinus has been associated with high caries potential (Badawi, Evans et al. 2003, Choi, Lee et al. 2009, Conrads, de Soet et al. 2014) and *in vitro* studies have shown that *S. sobrinus* is more acidogenic than *S. mutans* (de Soet, Toors et al. 1989, de Soet, van Loveren et al. 1991). Therefore, to potentially present a maximal bacteria-based biodegradation challenge, a *S. sobrinus* biofilm aging model was utilized in the present study. Contrary to what was expected, the 15-day *S. sobrinus* biofilm challenge produced lesser degradation of the adhesive interface resulting in higher μTBS value (23.58±7MPa) than those subjected to 15-day *S. mutans* biofilm (19.92±5MPa). Therefore, the third null hypothesis $H_{o}3$, which was related to the difference between the μTBS values obtained with these two bacterial exposures, was rejected. A preliminary experiment found a similar rate of drop in pH for broth cultures of *S. mutans* and *S. sobrinus* thereby refuting acidogenicity as a reason for the difference in their biodegradation capacities. A possible explanation for the variability could be that *S. sobrinus* has been shown to have lower potential for initial attachment and is aided by *S. mutans* in the same (Conrads, de Soet et al. 2014). Since single species biofilms were used in the present study, the absence of *S. mutans* or any kind of salivary facilitators could mean that fewer *S. sobrinus* than *S. mutans* organisms were in intimate contact with the dental materials.

A 14-day pilot study was conducted to determine the feasibility and appropriate sample size needed for the subsequent main experimental study. In addition to the water, *S. mutans* and *S. sobrinus* storage groups, we used brain heart infusion (BHI) broth adjusted to pH 4.5 as the fourth storage group. The latter group was chosen because it is a simple storage condition that represents the pH effect of cariogenic bacteria without having to grow the bacteria. However, it resulted in dark, spongy and excessively demineralized dentin substrate in the resin-dentin specimens. Although there were no

premature failures, the specimens stored under this aging condition had very low microtensile bond strength values. Therefore, the acidic BHI medium group was ruled out as a realistic clinically simulative challenge and was not included in the main experimental study.

BHI medium without sucrose was used in the main experimental study after the initial 24-hour sucrose-based inoculation. This was done because sucrose promotes adhesion of *S. mutans* and would have resulted in too great a biomass building up on the resin-dentin specimens during the remaining 14 days. A thicker biomass would not affect the glue-less mechanically passive gripping device used in this study but could create difficulties for laboratories that utilize active gripping by glue fixation for bond strength testing. Again, as one of our objectives is to encourage wide adoption of a biostability challenge to bond strength testing we chose a biofilm challenge that limits the biomass thickness. While there are some studies that advocate the use of sucrose or glucose to mimic the food intake and cariogenic challenge (Mutluay, Zhang et al. 2013, Li, Carrera et al. 2014), there are others that omit the sucrose (Bourbia, Ma et al. 2013). Borges et al. (2014) compared the effect of cariogenic (1% sucrose) and non-cariogenic biofilm challenge on microtensile bond strength of adhesives. They found no significant difference between the bond strengths of specimens exposed to either challenge (Borges, Kochhann et al. 2014). Therefore, it would be reasonable to say that our choice of eliminating sucrose after the initial colonization is not a factor/variable.

Failure Modes Associated with Types of Storage Conditions

Failure modes were evaluated under a light microscope and classified as adhesive, cohesive within dentin or composite and mixed. A chi-square test showed that there was a significant association ($p=0.0463$) between failure mode and the type of storage condition. The fourth null hypothesis H_{o4} is regarding the failure modes of the specimens

exposed to biofilm degradation and water storage. It was rejected because the resin-dentin specimens subjected to 15 days of a *S. sobrinus*- or *S. mutans*-based biofilm were 25 – 29 % more likely to have cohesive failures in dentin than specimens stored in water for 6 months. Also, a higher percentage of cohesive failure within dentin was observed in specimens stored in water for 5.5 months followed by 15 days of *S. mutans* challenge (74.2%) when compared to those stored in water for 6 months (54.8%). Hence, the fifth null hypothesis H_{o5} was also rejected. These findings are indicative of greater dentin degradation following biofilm exposure. Mutlay et al. (2013) also observed excessive dentin demineralization at the interface under Scanning Electron Microscopy (SEM). They postulated that *S. mutans*-based biofilm exposure led to greater demineralization of dentin at the hybrid layer-dentin interface resulting in failure under fatigue loading (Mutluay, Zhang et al. 2013). However, other studies (Borges, Kochhann et al. 2014, Li, Carrera et al. 2014) have reported increases in the probability of joint failures following a multi-species biofilm exposure. This could be attributed to the dissimilarity in the methodology. Nail varnish was used in the latter studies (Borges, Kochhann et al. 2014, Li, Carrera et al. 2014) to cover the dentin and composite substrates, leaving mostly the adhesive interface exposed to biofilm. Since dentin was left totally unprotected in the present study, the acids and enzymes in the storage medium might have damaged/attacked the outer surface of dentin substrate. Further, with a smaller surface area of the resin-dentin interface in the dumbbell-shaped specimens we might be able to obtain a similar aging effect in fewer than 15 days. This could prevent the excessive demineralization of dentin. The results of the present study supported the sixth null hypothesis H_{o6} related to the failure modes of the specimens exposed to *S. mutans*-based biofilm and *S. sobrinus*-based biofilm. 83.9% of the specimens stored in 15 days of *S. mutans*-based biofilm and 80.6% of the specimens stored in 15 days of *S. sobrinus*-based biofilm failed cohesively within dentin substrate and the difference was not statistically significant.

From the significantly lower μTBS values (19.26±6MPa) and greater number of cohesive failures within dentin observed (74.2%) in specimens stored in water for 5.5 months followed by 15 days of *S. mutans* challenge when compared to those stored in water for 6 months (49.69±15.53MPa and 54.8%, respectively) it can be hypothesized that 15 days of bacterial exposure magnified the hydrolytic degradation of resin-dentin interface produced by 5.5 months of water storage. However, further microscopic evaluation under higher magnification is required to confirm our hypothesis.

Statistical Analysis

In addition to ANOVA procedures, a Weibull regression model was also used for data analysis of the main experimental study. Weibull regression analysis accounts for the variation in the outcome (microtensile bond strength) while accounting for the clustering of samples, such as in the present case with four dumbbell specimens obtained from a single tooth (Hougaard 1995, Sahu, Dey et al. 1997, Armstrong, Keller et al. 2001b, Raposo, Armstrong et al. 2012). As discussed in the previous chapter, a disparity among the results was seen with ANOVA when tooth dependency (multiple specimens coming from the same tooth) was taken into consideration. However, with the Weibull regression model the same results were obtained in either scenario. These results reiterate the importance of using Weibull regression modeling for analyzing raw data when there are clustered samples. Also, the Weibull regression model adds the practical advantage of relating the probability of adhesive failure to the applied tensile stress and therefore it makes it possible to predict fracture probability at any level of stress (McCabe and Carrick 1986, McCabe and Walls 1986).

Summary/Advantages of the Study

When considering the totality of the results it is evident that biofilm storage produced significantly more degradation in just 15 days as compared to 6 months of water storage. Further, given the smaller surface area of the dumbbell-shaped specimens we might be able to obtain similar aging effects in even less than 15 days. The intentionally simplified biofilm-based aging protocol used in the present study is easy to repeat. Biofilm challenge can contribute to the standardized mechanical testing of bonding agents by expediting the aging process and evaluating the enzymatic stability of dental adhesives. Some might counter that biofilm-based aging is technically demanding and labor intensive. But the advantage of not having to wait months-years for clinically meaningful bond strength results outweighs the limitations and will encourage future researchers to adopt this type of aging model. In fact, *S. mutans*-based biofilm is increasingly being used in assessing the bonding performance of newer antibacterial resin monomers, such as 12-methacryloyloxydodecylpyridium bromide, MDPB (De Carvalho, Puppin-Rontani et al. 2014) and dimethylaminododecyl methacrylate, DMADDM (Zhang, Cheng et al. 2013, Wang, Zhang et al. 2014). Thus, the present study brings us closer to a promising *in vitro* aging method that is short-term and clinically simulative. However, if we were to do it again we might age the specimens for less than 15 days and plan to cover the surface of the resin-dentin specimens with wax. Alternatively, to simulate the *in vivo* scenario, we might expose the whole tooth-resin bonded assembly to biofilm challenge prior to sectioning it into resin-dentin sticks.

Limitations of the Study

From the results it is apparent that 15 days of *S. mutans*-based or *S. sobrinus*-based biofilm challenge produced more degradation of resin-dentin interface resulting in significantly lower μTBS values. Therefore, quantitatively the biofilm model is

successful in providing the aging effect in a short term of 15 days when compared to the standard protocol of using 6 months of water storage. However, there was an increase in the number of cohesive failures within dentin in specimens subjected to biodegradation. This is consistent with greater levels of demineralization and degradation of the dentin substrate. Greater number of cohesive failures within the dentin substrate may be considered a limitation of the aging protocol per se because as mentioned by Pashley et al. (1999) it prevents the actual measurement of the bond strength of the adhesive interfaces. They further explained that this type of failure does not necessarily indicate that the dentin was weaker than the adhesive-dentin bond (Pashley, Carvalho et al. 1999). Many factors, including the geometry of the tested interface, presence of adhesive flash, nature of load application and stress distribution has been shown to affect the bond strength testing (Van Noort, Noroozi et al. 1989, Van Noort, Cardew et al. 1991, Pashley, Sano et al. 1995). Cohesive failures within the dentin are suggestive of uneven stress distribution resulting in stress concentration and 'crack' formation within the dentin substrate (Pashley, Sano et al. 1995, Pashley, Carvalho et al. 1999). The dumbbell test specimen geometry and passive gripping device used in the current study have been shown to produce the most uniform stress distribution (Raposo, Armstrong et al. 2012). However, the uneven stress distribution could be a result of altered mechanical properties of the dentin due to excessive demineralization following a biofilm exposure. It has been proven that demineralized dentin has lower microtensile strength and elastic modulus than mineralized dentin (Sano, Ciucchi et al. 1994). On the other hand the failure modes observed following a cariogenic biofilm exposure in the present study relate to clinical failures. It can be postulated that the cariogenic bacteria, *S. mutans and S. sobrinus,* at the resin-dentin interface resulted in dentin demineralization and/or breakdown of ester bonds within the resin matrix. The biodegradation of adhesive interface may have led to ingress of bacteria, which in turn resulted in even more demineralization and degradation. The cycle of events in the study coincides with the clinically observed sequence of post-

op complications following composite restoration including marginal discoloration, marginal degradation and secondary caries (Deligeorgi, Mjor et al. 2001, Manhart, Chen et al. 2004, Spencer, Ye et al. 2010). Secondary caries has been cited as the most common cause for failure of resin-based composite restorations (Deligeorgi, Mjor et al. 2001, Manhart, Chen et al. 2004, Ferracane 2011). However there is controversy as to whether "secondary caries" is truly a primary caries lesion formed independent of the existing restoration or if it is a result of the bacterial microleakage along the 'marginal gaps' formed due to improper adaptation of the resin-based composite restoration. Consequently, a new term, 'caries adjacent to existing restoration,' is being commonly used in the literature (Mjor and Toffenetti 2000, Mjor 2005, Thomas, Ruben et al. 2007, Barata, Casagrande et al. 2012, Turkistani, Nakashima et al. 2015). Hence, there is limitation of not knowing how clinical failures occur. Also, we did not do a detailed investigation of our failure pathways and failure mechanisms. The failure modes were classified under light microscopy and without a higher magnification, such as with Scanning Electron Microscopy (SEM), it is hard to trace the exact pathway of crack initiation and propagation.

Remaining dentin thickness, calcium concentration and age of the dentin are some important dentin related factors that are known to impact the microtensile bond strength values (Sano, Shono et al. 1994). However, these factors were not accounted for in the present study. As noted by others (Hahnel, Muhlbauer et al. 2012, Borges, Kochhann et al. 2014), a simple *S. mutans*- or *S. sobrinus*-based aging model, like that used in the current study, does not simulate all the challenges of an intra-oral environment such as salivary flow, mechanical and thermal changes. Also, it does not incorporate the complex multi-species nature of dental plaque. When interpreting or comparing data from studies using a biofilm-based aging model, it is critical to consider the type of bacteria being used to challenge the dental bonding agent. As discussed earlier, bacterial species display different affinities and degradative effects towards various resin monomers.

Consequently, it may be more difficult to adopt a standard protocol using a live organism, especially since bacteria have a propensity to mutate or otherwise alter their characteristics when propagated long-term in laboratories. The biofilm-based aging protocol is labor intensive since it involves growing the bacteria, sterilization and changing the growth media every day with a precaution of avoiding contamination. However, the benefit of not having to wait 6 months to obtain similar aging effects far exceeds the limitation of being laborious.

Suggestions for Future Studies

To overcome at least some of the limitations of the present study, the dumbbell-shaped resin-dentin specimen can be coated with wax or nail varnish leaving a smaller region of the substrate(s) adjacent to the adhesive interface exposed to biofilm challenge. Alternatively, to simulate the *in vivo* scenario, whole tooth-resin bonded assemblies can be exposed to biofilm challenge prior to sectioning it into resin-dentin sticks. This method of aging also replicates the protective effect of the enamel around the dentin, a suggestion made by Borges et al. (2014) for future studies (Borges, Kochhann et al. 2014). To overcome the limitations with microtensile bond strength testing, a different test can be used in future. A mini-interfacial fracture toughness test has been recently introduced as a more valid alternative for microtensile bond strength testing. Higher probability of failures at the actual resin-dentin interface has been reported using this test. Instead of the dumbbell-shaped specimens, mini interfacial fracture toughness test requires the use of single notch resin-dentin beams (Pongprueksa, De Munck et al. 2016). Therefore, a future study can be designed using the mini-interfacial fracture toughness test with the biofilm-based aging model. To acquire further knowledge about biofilm-based degradation it would be useful to microscopically trace bacterial infiltration and study the fractured surfaces. As evident from the literature, Confocal Laser Scanning

Microscopy (CLSM) or Scanning Electron Microscopy (SEM) can be utilized for this purpose. In the present study only one bonding agent, Optibond FL was tested. This adhesive is a gold standard in the etch-rinse group of bonding agents. It would be interesting to additionally include a weaker adhesive as a negative control and compare the results to those obtained during clinical trials. Additionally this model could be used to test adhesives with and without antimicrobial (Imazato, Ma et al. 2014) or enzyme-inhibiting properties (Liu, Tjaderhane et al. 2011, Tjaderhane, Nascimento et al. 2013). Finally, different bacterial species such as *Streptococcus gordonii*, *Streptococcus sanguinis* or *Streptococcus mitis* that are commonly found in dental plaque can also be evaluated for aging of resin-dentin specimens. The rationale is to assess if cariogenic bacteria such as *Streptococcus mutans* or *Streptococcus sobrinus* are unique in producing biodegradation of resin monomers or these other non-cariogenic, acid-producing bacteria can also exhibit this property.

Conclusions

The findings of this study indicated that:

- 15 days of *Streptococcus mutans*- or *Streptococcus sobrinus*-based biofilm challenge produced more reduction in microtensile bond strength of dental adhesive than 6 months of water storage and appears to be a promising *in-vitro* aging model
- *Streptococcus mutans*- or *Streptococcus sobrinus*-based biofilm challenge resulted in higher number of cohesive failures within dentin than those observed following water storage. This is consistent with greater levels of demineralization and degradation of the dentin substrate following a biofilm challenge
- 15 days of *Streptococcus mutans*-based biofilm produced more reduction in microtensile bond strength of dental adhesive than *Streptococcus sobrinus*-based biofilm and therefore *Streptococcus mutans* represents a greater bacterial challenge for the dental adhesive tested in the present study

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