EFFICACY OF OVER THE COUNTER PRODUCTS IN REMOVAL OF *STREPTOCOCCUS MUTANS* FROM THERMOPLASTIC ALIGNERS – AN IN VITRO STUDY

by

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DEDICATION

I'd like to dedicate this thesis work to my family, my boyfriend, Nicholas, and my grandmother Elena. I have received unwavering support from my family in Bulgaria throughout my dental studies and my boyfriend, who supported me through my continuing postgraduate residency endeavors here in Ohio. My strength comes from knowing that I am making all of them proud.

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ABSTRACT

Background and Significance: The market for clear aligner therapy has been steadily expanding. Thermoplastic materials are commonly used to fabricate both clear aligners and retainers. Since the mouth is a septic environment, these aligners need to be cleaned periodically.

Purpose: The purpose of this study was to evaluate the effectiveness of five disinfectant methods commonly recommended to patients by their orthodontist to clean thermoplastic materials.

Methods: Molar segments cut from a popular commercially available thermoplastic aligners (Trutain, Rochester, MN, USA) were inoculated with *streptococcus mutans* and incubated for 48 hours in brain- heart infusion media to establish a biofilm on the thermoplastic segment. Segments were rinsed for 10 seconds with distilled water, dried and then cleaned with one of the following products. A commercially available foam product (WhiteFoam, Inglewood, CA, USA), a commercially available effervescent tablet (Retainer Brite, Centerbrook, CT, USA), dish soap (Dawn Dish Soap, P&G, West Virginia, USA) and toothbrush, white vinegar, or mouthwash (Listerine, J&J, New Jersey, USA), all according to manufacturer's directions. Following these cleaning procedures, a sample was taken from each aligner segment and plated on blood agar. Colony forming units were counted at 24 hours, and optical density was recorded at 24 hours and 48 hours.

Results: Agar plates with thermoplastic segments treated with foam, effervescent tablets, or mouthwash did not show presence of *streptococcus mutans* at 24 or 48 hours. Dish soap with

toothbrush and vinegar treatment showed significant *streptococcus mutans* colonies at 24 hours and 48 hours. Optical density readings at 24 hours and 48 hours showed continued increase in *streptococcus mutans* colonies for samples treated with dish soap with toothbrush, and vinegar, whereas samples treated with foam, tablets, or mouthwash showed no evidence of *streptococcus mutans* at 24 hours or 48 hours.

Conclusions: Antibacterial treatments were more effective than mechanical methods or vinegar for removal of *streptococcus mutans* from thermoplastic molar segments.

REVIEW OF THE LITERATURE

A YouGov study reported that 45% of adults have dissatisfaction when it comes to their smile and 20% would get orthodontics to correct appearances (www.bos.org.uk/news/ NOWYouGovSurvey) (Lombardo 2017). Due to an increased demand for esthetics within the last decade, patients have been walking through our doors asking for clear aligners. There is a public demand for more esthetic options to orthodontics and less metal devices (Jeremiah 2011). Aligners have gained popularity due to their near-clear nature and thus, invisible appearance (Rosvall 2009). The history of aligners gives itself to Dr. Kelsing (1945) who first used thermoplastic tooth positioners and validated this as a mode of orthodontic treatment (Low 2011). Dr. Kesling used close-fitting positioners to close small spaces after debanding teeth in the era of fixed banded appliances. Significant movements were hypothesized to be possible with sequential model set-ups (Low 2011). In 1997, Invisalign® (Align Technology, San Jose, Ca, USA) became the first to introduce computer-aided sequential clear aligners to predictably move teeth by small increments (Vlaskalic 2002). In the past, vacuum-formed materials have been used for retainers (Sikakakis 2018). The first Invisalign® aligner was fabricated from polyurethane and was recommended to be worn twenty to twenty-two hours per day, while removed during ingestion of food and oral hygiene practices (Boyd 2000). Abbate distinguishes that these aligners cover the teeth and marginal aspect of the surrounding gingiva and the removable fashion of these aligners allows patients to have better cleaning success due to having no obstructions such as brackets, archwires, or bands (Abbate 2015). Since the 1990's, Invisalign® material has evolved and so have other thermoplastic materials which are now used for orthodontic tooth movement.

Understanding Formation of Biofilm

To understand the biological process of oral disease, we must first dive into mechanisms of biofilm. Plaque, which can also be referred to as biofilm, is an intricate network of cells that attach to one another through extracellular polymers (Tektas 2020). The formation of biofilm is the result of chemical, physical, and biological interactions between a solid surface (i.e. enamel) and bacteria (Liljemark 1996). The formation of biofilm is a dynamic process that involves several overlapping phases.

The first is the formation of a conditioning film, also known as a pellicle, which is less than 1micrometer in thickness, taking 90-120 minutes to form (Marsh 1995). This occurs when enzymes such as glucosyltransferase (GTF), polysaccharides, lipids, and glycoproteins become entangled in tacky glucans, which the GTF enzymes synthesize (Steinberg 2004). The nutrients for plaque are these glycoproteins in saliva (Marsh 1995). Salivary products, such as amylase and proline then offer binding sites for the accumulation of bacteria (Schilling 1992). Bacteria considered 'early colonizers' are streptococcus and actinomyces varieties such as *A. naeslundii, Streptococcus mitis, S. gordinii, and S. sanguis* (Marsh 1995). Kolenbrander et al 1993 describe Fusobacterium as a species that are capable of co-aggregating with a plethora of other genera but incapable of co-aggregating with itself. Fusobacterium, such as *F. nucleatum*, act as the bridge between the early and late colonizers (Kolenbrander 1993). At this stage, bacteria will synthesize from sucrose, extracellular polysaccharides (EPS), which consist of glucans. This EPS will interact with receptors in the pellicle and provide structural integrity (Marsh 1995).

As different species of bacteria accumulate, the biofilm matures. It is important to note that bacteria do not move, and is not motile. It is not capable of directly adhering to tooth structure (Marsh 1992). It is transported passively by saliva and the tongue, and indirectly adheres to the tooth through the pellicle (Tetkas 2020). Therefore, this process of maturation depends on the

movement of secondary bacteria onto primarily attached bacteria, a process known as co-aggregation (Marsh 1995).

Two to four hours after pellicle formation, it is common to see initial colonizers; mainly gram-positive cocci. In one to two days, the flora consists of gram-positive rods and filaments extending from the cocci substructure (Marsh 1995). As the depth of the biofilm increases, the oxygen tension is decreased, thus producing a substantial increase in the anaerobic species (Nyvad 1993). Additionally, EPS content of the plaque will allow sugar to permeate deeper into the plaque, increasing its cariogenicity by plummeting the pH of the biofilm (Dibdin 1988). Zero et al studied the ratio of EPS to actual bacteria in a demineralized enamel lesion which he found was 95% and 5% respectively, truly showing the acidity and damage caused to enamel. In plaque, bacterial cells are in close contact, and will degrade the host (enamel), block salivary mucins, and produce inhibitory factors like hydrogen peroxide (Marsh 1992).

Two to three weeks of undisturbed plaque formation will result in what is referred to as a 'climax community', 50-100micrometers in thickness and typically not on smooth surfaces. The surface that this plaque is likely to grow on is irregular, such as interproximally, inside of deep pits and fissures on the occlusal surface of the tooth, or inside the gingival crevice (Marsh 1995). It can consist of upwards of 700 different microbial species, including aerobic and anaerobic species (Tektas 2020).

Why is understanding biofilm important? *Streptococcus mutans* and *streptococcus sobrinus* have been pinpointed as crucial bacteria in proliferation of biofilm in the oral cavity. Specifically, their ability to cause disease is due to their high ability to adhere and thrive in acidic environments, making them key players in dental disease. (Liljemark 1996) Low et al (2011) grouped subjects in categories of "slow" and "fast" plaque formers. Plaque indices were measured in vivo, and subjects were instructed not to brush for 48 hours. The 'slow' plaque

formers had a mean plaque score of 13.1% and 'fast' plaque formers 63.7%. Low et al looked at each aligner under scanning electron microscopy (SEM) to observe the rate of which plaque grows and how important topography is. He shows no difference at hour 6 between slow and fast plaque formers. Hours 6-12 showed coccoid colonizers were evident on corrugated portions, cracks, and grooves of the Invisalign® aligner. It was demonstrated that 24-28 hours, there was more evidence of rods and filamentous forms in addition to the cocci, encased in EPS substance (Low 2011). In fast plaque growers, these biofilm complex communities were larger, and more voluminous in the 3-14 day samples. 48hr-14 day also samples revealed an addition of crystalline structures (i.e. calcifications) (13 Low 2011). This study reveals a clinical example of plaque development in every stage under extreme magnification (x845). Quirynen and Bollen (2005) have also confirmed that as plaque ages, it increases in amplitude of surface roughness. The topography of the aging plaque communities furthers the niche for bacterial growth, in which bacteria become more protected. At this time, plaque becomes an irreversible biofilm, and unable to be disturbed by oral hygiene and forces of food mastication (Quirynen 2005).

A bacterial special continues to morph through the phenomenon called 'quorum sensing'. Bacterial cells have cell-cell signaling systems that can help them thrive by evolving through environmental stresses (Marsh 1995). Quorum sensing may contribute to the pathogenicity of bacteria through expression of genes (Marsh 1995). *Streptococcus mutans* mediates quorum sensing by a protein by the name of competence stimulating peptide (CSP) (Marsh 1995) and this is shown to be used in acidic environments to express a protective response (Li 2001). Plaque does not only form on tooth substance. Foreign substances, like orthodontic brackets and aligners also develop bacterial plaque (Eliades 1995). Microbial biofilms have been estimated to be a causative factor in 60% of human infections such as dental caries, periodontal disease and candidiasis (Costerton et al 1999, Marsh 1995). Introducing orthodontic aligners limits the

natural ability of the oral cavity to buffer and remineralize from the salivary proteins and thus, biofilm on the enamel and intaglio surface of the aligner are not able to be washed away by the mechanical disturbance of the lips, tongue, and cheeks (Lombardo 2017).

Dentistry itself lends itself to play a role in biofilm formation. We integrate foreign materials into the mouth with dentistry which are of different texture; for example crowns, dentures, brackets, and aligners. When a new material is introduced, the opportunity for biofilms to form is endless. These biofilms are unique to the specific environment that they live in. Changes in the surface roughness, morphology, and oxygen availability dictate a unique flora to form and perpetuate into unique matrices for the bacteria (Bowden 1997).

Orthodontic Aligners

Tuncay et al (2013) investigated orthodontic treatment with aligners specifically in teenagers and results of biofilm induced pathology. The investigators looked into plaque index score of 42 teenagers (male=female) which were deemed ‘compliant’ in wearing their aligners and formed statements on how aligners may assist in the business aspect as well. Researchers found that plaque index on the maxilla versus the mandible had no significance, 15.1% and 16.6, respectively. Results showed that one-third of the enamel surface on maxillary molars were covered in plaque, indicating that this site is the worst in the mouth for plaque buildup. Most importantly, Tuncay et al were able to show that there was only a 0.1% increase in plaque index within the first 3 months of treatment with orthodontic aligners. This is compared to similarly conducted research using fixed appliances, and researchers claim that patients wearing removable appliances had significantly better oral conditions (Tuncay 2013). Klukowska et al (2011) in a similar study, reports a 57% plaque coverage in the anterior gingival region and 68% coverage around orthodontic brackets and draws the conclusion that gingival margins were

difficult to scrub in the presence of fixed appliances (Klukowska 2011), which Tuncay claims is a ‘non-issue’ with patients wearing removable aligners. A patient report reveals that 70% of patients wearing aligners during this time reported “seldom or never experienced discomfort from the aligners’ and 80% ‘seldom or never used pain relievers’. This study indicates that teenagers are able to be comfortably treated orthodontically with aligners, there is adequate treatment for success of movements, minimal change in plaque index along treatment and favorable sequela for the health of the gingiva compared to that of fixed appliances. Ergo, clear aligner therapy is advantageous due to the aligner esthetic appearance, better dental and gingival health, and ability to remove them for eating and access to oral hygiene.

Orthodontic Aligners vs Fixed Braces: Periodontal Disease

Periodontal disease is a common disease caused by bacteria. Shifts in the microbial biofilm community have an altering effect in the relationship of host and microorganism. It is important to understand these changes to avoid disease related to orthodontic appliances. The accumulation of plaque leads to gingival inflammation. This inflammation augments the flow patterns of gingival crevicular fluid which intern, feed plasma proteins (Marsh 2009). These plasma proteins are responsible for anaerobic bacterial growth (Marsh 2009). Tuncay’s study (2013) opens up the question if the selection of methods of moving teeth has consequences. According to this study’s conclusions, is it safe to say that fixed appliances cause periodontal disease or in the least, inflammation. If this were true, then orthodontic appliances would be considered risk factors for a patient's enamel. Steinberg (2004) looked into initial biofilm on brackets, modules, coil springs, and elastics. They found that modules (highest) and brackets (second highest) had great affinity for salivary proteins, which were most contaminated by specifically radioactive labelled *streptococcus sobrinus* (Steinberg 2004). However, it has been

shown that aligners do also facilitate biofilm deposition and they do indeed contribute to worsening of oral health. (Karkhanechi 2012)-- however in contrast to fixed treatment, patients treated with aligners have a better periodontal status and less pathologic anaerobic bacterial counts. There is no doubt that being able to remove an appliance produces a more efficient ability to clean teeth. Karkhanechi et al (2012) demonstrated that plaque index, bleeding on probing and periodontal pocketing was superior in patients wearing aligners when compared side by side to fixed appliance wearers. The opposite was evidenced by Guo et al (2018), who extracted DNA from plaque samples and looked at periodontal parameters like plaque index and gingival bleed index in female patients wearing clear aligners for three months. Guo et al did not see a statistical significance in the affluence of periodontal pathogens at the 3 month mark (Guo 2018). Throughout treatment, there was a decrease in the diversity of the biofilm on aligners, although all patients were free from periodontal disease and had normal levels of periodontal and core microorganisms (Guo 2018). Researchers concluded that only non-pathogenic changes in the subgingival microbiome were induced in those first three months in female patients and suggested that perhaps instead of periodontitis, we should be concerned of gingivitis (Guo 2018). Rossini et al found that patients treated with clear aligners have a superior or at least equal periodontal health as those with metal braces (Rossini 2014). A study by Meithke and Vogt (2005) also corroborated this in their study with patients and Invisalign® therapy and fixed therapy. Results indicated that patients wearing Invisalign® did not have statistically significant probing depth increases during treatment and are no more vulnerable to disease of the periodontium than with patients wearing fixed. They compared the oral hygiene of both groups and observed an improvement in the Invisalign® group, due to an older and more motivated patient pool as well as the aligners being able to be removed. (Meithke 2005).

In a study measuring full mouth plaque and bleeding scores, it was found that these scores both tripled in teens wearing fixed appliances, whereas both scores fell in teens with Invisalign® (Abbate 2015). Compliance with respect to oral hygiene were observed as well, showing this was significantly higher in Invisalign® teens (Abbate 2015). Plaque Index, probing depths, and bleeding on probing was measured at the mesiobuccal of the upper right first molar and upper left central incisor and with <0.01 certainty it was significantly lower in the Invisalign group (Abbate 2015). Conclusions of this study encourage clinicians to determine individual teens oral hygiene, and consider aligner therapy, as there is less plaque and inflammation in Invisalign® wearing teens compared to their fixed appliance peers (33 Abbate). In observance of plaque and gingivitis scores by Sifakakis et al (2018), these periodontal parameters were statistically significant for thermoplastic aligners being favored (Sifakakis 2018). This agrees with previous findings.

Levrini et al (2015) also studied differences in Invisalign® and fixed appliances. Biofilm mass was scored as well as bleeding on probing, probing depths and plaque index. In the Invisalign® group, periodontal parameters were all low, and total biomass was statistically significant in favor of Invisalign®; even in a 90 day follow up (Levrini 2015). Levrini et al conclude that with fixed appliances, there is a higher quantity of retention sites that can have potential to lead to periodontal inflammation (Levrini 2015). Furthermore, there is an absence of periodontopathic bacteria in the Invisalign® group, aligners allow for oral hygiene procedures, and PCR evidence of higher bacterial counts in patients with fixed appliances versus aligners (Levrini 2015).

Periodontal disease or simply, gingival inflammation can be attributed to a shift in the microbial composition due to food entrapment (Ristic 2007) and in the absence of brackets, bands, and wires, oral hygiene is attainable (Levrini 2013; Abbate 2015). Levrini et al asserts that clear

aligners should be the first choice for patients that have possessive periodontal problems and or in adult patients (Levrini 2015).

These studies help us focus on differences in periodontal status and access surely is important in being able to clean effectively. Along with caring for the teeth themselves, it is important to clean the aligners themselves, as they can retain plaque accumulation within the thermoplastic material itself. Cleaning methods will be discussed further.

Periodontal Disease

It is persistent plaque that causes inflammation. We know now that the maturation of dental plaque introduces risk by allowing anaerobic bacteria to thrive within the deep thickness of the biofilm, which largely can introduce a potential for periodontal pocket formation (Thurnheer 2015). The periodonto-pathogenic bacteria are largely anaerobic (Thurnheer 2015). Aligners seat to the gingival margin for extended periods of time, leading Schaefer et al (2010) to study oral health, periodontal health, and halitosis and test the claim they are considered a periodontal risk (Schaefer 2010). Observing sulfur compound levels after patients used chlorhexidine 0.06%, they found that low volatile sulfur compounds significantly decreased, and that halitosis, dryness of the oral cavity, were unaffected in this group. This concludes that despite full coverage to the gingiva, Invisalign® treatment only minimally impairs the health of the oral cavity and there is a benefit for the gingival health if 0.06% chlorhexidine is implemented in the regimen (Schaefer 2010).

The Caries Process

Along with periodontal health, researchers have investigated the effect of aligners with carious lesions. Dental caries is a microbiological infectious disease that translates to destruction

of once calcified tissues (Somasundaram 2013). Caries formation is caused by a disturbance in the normal cycle of demineralization and remineralization (Yadav 2012). Caries may be reversible or irreversible, and studies have shown that this is a worldwide issue in public health in all areas from rural to urban (Joshi 2013). White spot lesions are the very first manifestations of caries progression and are still reversible (Vashisht 2013) and are an unaesthetic common finding when orthodontic treatment is performed (Oogard 1988).

Opaque, white spots on the enamel surface are early carious lesions, which are characterized by color, and are slightly softer than sound enamel on the periphery (Oogard 1988). The color is due to an optical phenomenon and it will typically increase in quality of the white color as the surface of the tooth is dried. A few studies exist that use technology to quantify the surface of enamel, those being namely polarized light microscopy, microradiography, microhardness, and electron microscopy (Oogard 1988). Carious enamel forms in two different observed ways. Surface softening begins by preferential dissolution of interprismatic substance and loss of mineral is marked at the surface of enamel. (Oogard 1988) The second is by a subsurface lesion. This dissolution occurs in the deeper portion of the enamel, where a mineral rich layer (although porous) covers the lesion, which is low in mineral content. Kinetic events are attributed to the relatively intact mineral rich later and porous surface layer. (Oogard 1988).

Because aligners are in the oral environment a large portion of the day, covering the teeth will obstruct the ability of saliva to cleanse tooth surfaces, missing it's remineralising effects (Lombardo 2017). Without orthodontic appliances in place, the lips, tongue, and cheeks also play a role in disturbing biofilm formation on the teeth (Lombardo 2017). Therefore, is there a concern with aligners causing demineralization?

Streptococcus mutans has been blamed to assist in development of dental caries (Nyvad 1993) and its colonization depends on presence of hard surfaces, namely, after tooth eruption. (Tenuvuo 1991). The highest isolation of *streptococcus mutans* has been found at the end of exfoliation of deciduous teeth because interproximal contacts and fissures are tighter, especially in the permanent molar sites (Tenovuo 1991). The earlier MS colonization has been found; the higher prevalence of caries has been found. (Kohler 1988, Schroder 1987). As a caries lesion advances, a change in the microflora occurs. The deeper part of the carious lesion typically is infiltrated with lactobacilli because acidity is highest here (Nyvad 1993).

Several factors are to be considered for creating an environment of decalcification. Oral hygiene, exposure to fluoride, diet, saliva flow can all influence the biofilm formation and prevalence of caries (Tenovuo 1991). Identifying these factors for orthodontic patients may allow orthodontists to effectively identify patients at higher risk for development of demineralized enamel.

Several studies have found that the presence of orthodontic appliances does have a ramification on the microflora, both quantitatively and qualitatively (Petti 1997, Scheie 1984). Batoni et al (2001) found that the appliances that are removable can create new retentive surface areas that promote streptococcal adherence (Batoni 2001). In this experiment, researchers observed caries risk for patients in a control (not wearing removable orthodontic appliances) and ones wearing orthodontic removable appliances. Colonization of *streptococcus mutans* in dental plaque was higher in the group of children wearing removable appliances with statistical significance. Batoni et al concluded that children representative of the aligner wearing group, should be monitored carefully for caries formation during treatment. There are numerous variables in the causation of caries, namely frequency of brushing, acidogenic potential of the strain of *S. mutans* present in their biofilm specifically, and sugar consumption while wearing aligners (Petti 1996, Petti 1999). The importance of *streptococcus mutans* is underlined in the

oral environment because the glucans and GTFs that this bacterium secretes bind avidly to the surface pellicle. When met with sucrose, glucans will form in only minutes (Mohsin 2015), which encourages the bacterial accumulation and therefore, its virulence. There are more than 30 species of bacteria per milligram in net weight of plaque (Hwu 2014), therefore enumerating the importance of control of bacteria on tooth surfaces.

For centuries we had no technique to remineralize decalcified enamel other than surgically removing them and providing a restoration on top. There was simply no other alternative to treating this type of enamel until about one century ago. Mittal et al (2017) investigated a variety of enamel remineralization techniques and broke down the benefits of each (Mittal 2017). Fluorides, casein calcium phosphopeptides, bioactive glass agents and others exist commercially, each playing a different role in the remineralization of tooth tissue. Fluoride is the most used agent for remineralization (Mittal 2017). After an acid attack on teeth, the pH begins to rise and uses fluoride crystals containing fluorohydroxyapatite in order to discontinue the dissolution of enamel (Lata 2010, Cate 1990). According to Khan et al (2002), fluoride is capable of remineralizing an incipient lesion, interfering with the formation of biofilm and its organisms, and increases rates of tooth maturation post-eruption (Khan 2002). The efficacy of fluoride dentifrices is best at 5000ppm, as evidenced by Jo et al (2014). Casein phosphopeptide-Amorphous calcium phosphate (CPP-ACP), also known as Tooth Mousse GC, was first formulated in 1998 for its properties of anti-cariogenicity utilizing milk and cheese (Reynolds 1997). Phosphorylated caseins (CPPs) with a cluster of amino acids -ser(P)-ser(P)-ser(P)-Glu-Glu binds and stabilizes phosphate and calcium, as well as dental plaque and enamel. Each CPP will bind 15 phosphate ions, 5 fluoride ions, and 25 calcium ions (Vashisht 2010). Amorphous calcium phosphate (ACP) will be bound by CPP and will prevent the dissolution of calcium and phosphate ions, holding on to calcium and phosphate as a reservoir and creating an environment

for remineralization (Reema 2014). Bioglass, also known as NovaMin (registered mark), is an amorphous calcium sodium phosphosilicate and is known to release phosphate and calcium in the oral cavity to repair dissolved enamel (Mittal 2017). A silica layer forms via precipitation of ions that is capable of forming a hydroxyl-carbonate apatite, and it is used as a demineralization agent, although is proven to promote apatite formation through chemical reactions (Mittal 2017). Lastly, Mittal et al described xylitol to be 'tooth-friendly' type of sugar alcohol. It is used as a sweetener in foods and is considered to promote less biofilm formation due to its inability to ferment acids (Reema 2014). This will ultimately decrease the amount of S. mutans in the environment and displays that xylitol is involved in remineralization by carrying calcium required for this process (Hemagaran 2014).

If aligners may be responsible for harboring bacteria that assists in dissolution of enamel, there need to be tools to help prevent these biological processes from happening, whether that biofilm is on the tooth surface or on the intaglio of the thermoplastic aligners. The above-mentioned methods have different mechanisms of actions and can all be considered in remineralization for white spot lesions (Mittal 2017). Numerous studies have investigated the difference between aligner orthodontic therapy and fixed orthodontic therapy and assessed the incidence of caries and/or white spot lesions.

Aligner Therapy vs Fixed Braces: Caries & White Spot Lesions

Aligners may have an ability to contribute to the formation of decalcification and white spot lesions, and several articles compare the adhesion of bacteria in removable thermoplastic aligners versus a traditional fixed bracket orthodontic treatment. Claims made by Mitchell et al (1992) suggest that orthodontic fixed brackets create a specific change in the oral cavity that will decrease pH along with increasing plaque adhesion and electrostatic reactions with the metal

surface (Steinberg 2004, Mitchell 1992). This very concept is what we will investigate in this section.

White spot lesions in regard to fixed orthodontic treatment have been thus discussed; however, no we have not discussed orthodontic bands. Ogaard et al (1988) published an article that put orthodontic bands under scrutiny. Researchers examined plaque accumulation on ill-fitting orthodontic bands, as they were used frequently historically in orthodontics. According to their results, lesions developed facially, on anterior and posterior teeth. White spot lesions are capable of forming in 4 weeks (Oogard 1988). This is typically the period between appointments in real world orthodontics, so careful inspection of all banded appliances on teeth are recommended to be done at every orthodontic appointment. The author also recommends having a preventative fluoride regimen to follow with all patients. Fortunately, banded orthodontics is no longer used in contemporary orthodontics, however they still have a place in fixed appliances. This recommendation is still applicable in modern day orthodontics, as banded appliances are still in use. Irrespective, the information that we can take from this is that any bracket or band we place must be monitored at every orthodontic visit (Oogard 1988).

17 girls and 13 boys with a mean age of 13.8-year-old were placed into groups, either to be treated with self-ligating appliances or clear PET-G thermoplastic aligners. Saliva was sampled as a baseline, 2 weeks into treatment, and 4 weeks into treatment. Plaque index, gingival index, and a DMFT index, counts of *streptococcus mutans, streptococcus sanguinis, and lactobacillus acidophilus* were clinically assessed. The results of this investigation showed that aligner wearing patients had lower plaque index and lower gingivitis scores compared to all timepoints of those treated with fixed appliances. However, despite this finding, no statistically significant difference was found between the groups in counts of *streptococcus mutans* counts. *Streptococcus sanguinis* was significantly lower in aligner wearers, and there was almost no L.

acidophilus present in any of the saliva samples from either test group (Sifakakis 2018). There was no difference in factors such as age, gender, DMFT, and salivary flow. It is important to highlight that *Streptococcus sanguinis,* along with *Streptococcus mutans* are both found as key players in formation of dental caries, and both being present causes an environment for tooth demineralization (Babaahmady 1997). This opens a discussion for what exactly happens with biofilm formation on brackets.

One would believe that orthodontic brackets provide a plethora of places for pathogenic bacteria to adhere. Papaioannou et al (2007) investigate the adherence of *Streptococcus mutans* to different types of brackets, namely stainless steel, ceramic, and plastic brackets. There was shown to be no difference in the counts of cariogenic streptococci in these raw orthodontics materials (Lim 2008). The material in which the brackets are being fabricated do not increase or decrease bacterial counts (Papaioannou 2012). However, it was later discovered that the bonding adhesives have more to do with the cariogenic streptococci species adhesion than the bracket itself (Lim 2008). Babaahmady et al (1997) shows that resin-modified glass ionomer adhesives have the greatest interaction biofilm formation. Lightbond, Transbond, RMGI, stainless steel, polycrystalline alumina, monocrystalline sapphire, and hydroxyapatite block were tested as raw materials, with RMGI scoring with most *S. mutans and S. sanguinis* affinity (despite its fluoride releasing properties), and stainless steel brackets and polycrystalline alumina brackets being with lowest affinity to both of these bacterial species (Lim 2008). This can be explained by the coating or finished surface of brackets, whereas bonding adhesives are irregular and rough on the surface; ultimately this rough surface introduces nooks and attracts bacteria (Lim 2008). In the study by Sifakakis et al (2018), the thermoplastic wearing group did not include attachments to the teeth. This study was designed with no attachments due to previous evidence that

attachments introduce more plaque retentive surface, which typically would be a light cured composite, whether it is a bulk, hybrid, or flowable composite material (Lim 2008).

Another study by Buschang et al (2018) considered incidence of white spot lesions on clear aligner and transitional braces wearers. It was found that 1.2% of aligner patients developed white spot lesions. This finding is significant compared to the 25.7% traditional fixed bracket therapy patients that presented with white spot lesions (Buschang 2018). The study also posed the question; where do we see the most white spot lesion development? 244 aligner patients and 206 fixed appliances were chosen, and photographs pre and post treatment were observed for white spot lesions. Gender and pre-treatment white spot lesions were not statistically different. The location in the mouth where highest white spot lesion incidence was found was the maxillary teeth (Buschang 2018). Anteroposteriorly, more white spot lesions were found in the canine area, followed by the lateral incisors, and lastly the central incisors (Buschang 2018). Greater proportions of participants in the fixed braces had classifications of 'poor' OH. There were three patients of the 244 aligner patients who developed white spot lesions-- two in the maxillary arch and one in the mandibular arch. In contrast, of the 206 fixed appliances patients, 52 developed one to twelve new white spot lesions (Buschang 2018). The results suggest that with fixed appliances, a higher white spot lesion count is to be expected due to greater bacterial accumulation (Naranjo 2006) and counts, and challenges with cleaning the crevices of brackets. (Rosenbloom 1991). Pre-treatment poor oral hygiene was linked to 6.5 times more chance of developing a white spot lesion with fixed bracket therapy; suggesting there is a link between pre-treatment oral hygiene and development of white spot lesions (Moshiri 2013). Lower incidence of white spot lesions in aligner patients was attributed to better access for OH and shorter treatment duration times (Buschang 2018). Treatment duration with aligners was 0.9years shorter than the traditional group (statistically significant). Treatment duration past 36 months with fixed

appliances increased the risk of developing white spot lesions (Buschang 2018). This study was conducted with private practice records, which may have introduced bias into the study.

Tuncay et al (2013) as well as periodontal parameters, to a smaller degree than Buschang et al, observed 336 teeth for decalcification in aligner treatment and found that 75.6% of teeth exhibited no point change, 14.3% exhibited one point change, 0.9% (3 teeth) showed an increase by two points and another 0.9% (3 teeth) showed a range of three points. The point systems are as follows: "0 means no white spot lesions present, 1 means visible white spots without surface interruption, 2 means visible white spots with roughened surface but requiring no restoration, 3 means visible white spot lesion with surface interruption, and 4 means cavity" (Tuncay 2013). Researchers state there is virtually no evidence of enamel surface lesions, perhaps meaning actual cavitation. Concluding this study, Tuncay et al (2013) find a potential opportunity in orthodontics for treating young patients (with aligners) who may not go through with orthodontic treatment. These findings are far more favorable than the previously reported findings by Chapman et al (2008) who reported 1 or more white spot lesion in every traditional appliance wearing patient, and statistically significant in the maxillary incisors, canines, and first premolars.

In a very similar study design as Buschang et al (2018), Chapman et al (2008) observed 332 Indiana University patient records and outlined white spot lesion development in maxillary teeth in only patients wearing fixed orthodontic appliances. He found no significance to gender, although more white spot lesions were discovered in female patients in this study. This is interesting because females have shown to express more careful interest in their oral health, where brushing and flossing their teeth happened more frequently (Sakki 1998). Chapman et al (2008) found that males had twice the size of white spot lesions than females, and appeared in 46% of males, whereas 29% of females developed white spot lesions. Contrary to Buschang et al

(2018) findings, Chapman et al (2008) reports the highest incidence of white spot lesions appear to be the laterals, followed by the canines, first premolars, second premolars, and lastly central incisors; with no differences in white spot lesions were noted right side to left side. The average size of the white spot lesions observed occupied 9.8% of the facial tooth surface (Chapman 2008). Bracket type varied in the sample with 87% being conventional metal brackets, 8% ceramic brackets, and 17% self-ligating brackets. The mean treatment time was 32 months. The conclusion of this study was that at least one white spot lesion appeared when looking at the eight maxillary teeth (Chapman 2008), and this was decreased from previous studies which reported 49.6%. The studies may have had a different standardization in examination and there may have been differences in banded vs bonded appliances used historically (Gorelick 1982). The take home message from this study was that younger patients have been known to have a higher risk of white spot lesions. This may suggest that older patients are more fitting patients for fixed orthodontics (Chapman 2008). Brushing habits and duration of treatment, especially in a case with two-phases, are strong factors to consider when selecting fixed appliances or aligners.

Halitosis

Among periodontal disease and the carious process as a concern for aligner wear, one must also discuss the point of halitosis; also known as 'bad breath'. It has been suggested that 85-90% of halitosis cases are related to an oral cause (Shaefer 2010). Volatile sulfur compounds such as hydrogen sulfide, dimethyl sulfate, and methyl mercaptan are all increased when halitosis is observed (Miyazaki 1995). These three compounds are fermented by gram negative bacteria in the oral cavity, and mostly from the dorsum of the tongue. There are two different types of halitosis; one that is 'true' which is in fact, active halitosis, and there is a pseudo halitosis, which is subjective only (Shaefer 2010). There is a strong correlation between these

two types of halitosis and the sensation of dry mouth, because the salivary flow is reduced when the mouth is dry. This causes a shift in the microbiological equilibrium, and this is when we can start to see an increase in the volatile sulfur compounds (Shaefer 2010).

Invisalign® patients have historically described their subjective, or 'pseudo' halitosis, feeling like their mouth is dry. Given that Invisalign® aligners are meant to be worn for 22 hours per day, halitosis is a concern (Shaefer 2010). Chlorhexidine rinse has been used for years for its antibacterial bactericidal and bacteriostatic properties (Addy 1997). Chlorhexidine has been shown to improve halitosis by reducing the volatile sulfur compounds present in the oral cavity, mainly in the dorsum of the tongue, as evidenced by Nachnani et al (1997). Knowing these findings, Schaefer et al (2010) performed a crossover study in which 31 patients in two 3 months groups. Participants in group 1 used a low dose 0.06% chrlohexamed mouthrinse and nothing in the second 3-month period; and the second group did the opposite. Stimulated salivary flow rate, organoleptic index of malodor, and tongue coating index, volatile sulfur, gingival index, bleeding on probing, and plaque index were measured for each patient. Using a 5% level of significance, the organoleptic index, tongue coating index, plaque index, gingival index, bleeding on probing, or stimulated saliva flow rate were all not statistically significant (Shaefer 2010). However, the halimeter readings on the first group to use chlorhexidine indicated there is a positive effect when using chlorhexidine with a $p=0.03$. An interesting finding was observed in which the number of participants who cleaned their teeth more than three times a day doubled in the duration of this experiment, and the average amount of time spent with oral hygiene also increased (Shaefer 2010). It was suspected that participants who fell into the 'pseudo' halitosis category are simply more perceptive, or aware, of slightest change in the mouth (Shaefer 2010). In summary, there was no concrete evidence of halitosis, dry mouth, or inflammation with

Invisalign® in the first eight months of treatment; however patient oral health was stressed and participants may have been hyper aware of their oral care needs, not fully creating a real-life scenario. It was not evidenced in this study that chlorhexidine absolutely needed to be implemented with aligner treatment (Shaefer 2010).

Properties of Plastic

Resin polymers are what make up aligners. The very first aligner was created only from one layer of polyurethane sheet (Lombardo 2015), followed by Invisalign® Exceed-30 aligners, which had superior transparency and fracture resistance. Thermoplastic polyurethane (TPU) is what is currently used by Align technology, and is multilayered, coining the term "smart track" aligners (Lombardo 2015). This material, according to the manufacturer, has a lighter structure and has the ability to place continuous light forces onto the teeth with greater elasticity, and can provide superior predictability of tooth movements. Other non-Invisalign® aligners are being fabricated with the use of polyethylene teraphthalate glycol (PETG), that is a transparent, amorphous copolymer that is non-crystallizing and no strain-induced crystallization. This material historically was used more for orthodontic retainers (Lombardo 2015) Other aligners could be fabricated by polypropylene, ethylene vinyl acetate, and polycarbonates. Thickness of aligners vary between 0.5mm and 1.5mm (Lombardo 2015). It is worth mentioning a new aligner material called F22 (University of Ferrara; Italy) formed from a chemically inert polyurethane that has supposedly enhanced properties such as transparency, resistance to stress and malleability (Lombardo 2015).

In-vitro studies have been conducted to test what type of forces would be applied to teeth. These in vitro studies revealed that pressure formed aligners deliver more forces than vacuum formed aligners, and at a higher rate of activation (Eliades 2017). The anatomy of a crown may result in

an inadequate or uneven rate of force on different surfaces of the tooth, which may alter the success of precise movements needed. It was concluded that the force systems are determined by the position of the teeth and their adjacent teeth (Eliades 2017). This is potentially a limitation in the study of force because in-vitro studies do not simulate factors such as the periodontal ligament.

Because aligners are formed from resin polymers which are not inert (except for above mentioned F22 aligner material), they are not resistant to changes from temperature, forces from mastication and long-term contact with salivary enzymes. (Lombardo 2015). Wear experiments have been previously performed on aligner materials to define their properties. The term hardness shows wear resistance. It was shown in (Eliades 2017 -15) that PETG materials perform better in wear resistance trials compared to two different polypropylene aligner materials in this cyclic wear study. Another characteristic is the modulus of elasticity. An appliance that has high modulus of elasticity is able to be fabricated thinner and still delivers forces to the teeth. It was shown that Invisalign® has a higher modulus of elasticity than PETG material, which makes it slightly more brittle. Creep is one more characteristic worth mentioning. The higher an indentation creep, the more likely that the aligners will deform and potentially thin from orthodontic forces prescribed; Invisalign® has the higher creep than PETG counterparts. This study integrated these properties in an in-vitro environment. This information does not necessarily mean that Invisalign has superior results and in-vivo studies are required to test these characteristics with intraoral factors.

Invisalign® movements were compared to fixed appliances movements. Invisalign® had some pros: closing spaces, correcting rotations in anterior segments, marginal ridge heights. Cons were anteroposterior changes, occlusal contacts, and posterior torque (Eliades 2017 - 23). Kravitz et al (Eliades 2017- 22) found that the mandibular canine was the most difficult tooth to

control, the most accurate tooth movement was lingual constriction, and least accurate tooth movement was extrusion. It was demonstrated by Eliades (2017) that there was more evident relapse in Invisalign® cases than with conventional fixed appliances.

What happens to aligner materials after they have been used for one week? Salivary enzymes, warmth, humidity, biting forces can create changes in the composition of an aligner (Eliades 2017). It is difficult to simulate exactly the oral environment, however studies have attempted this. Polymers absorb water and this causes expansion and changes in mechanical properties of an appliance. Hygroscopic expansion may ultimately affect the fit of the aligner and the delivery of orthodontic forces. Eliades (2017) found that two weeks submerged aligners absorbed water in several different materials, and Invisalign® demonstrated the highest water absorption, closely followed by one type of PETG aligner. Absorption is not the only issue, however. Invisalign® worn for 14 days show delaminated areas, microcracks, and calcified plaque deposits as well as loss of transparency (Eliades 2017). Surface roughness increased over six-month intraoral exposure of a PETG vacuum-formed material (Eliades 2017). Aging of this material showed increased hardness and changes in molecular composition, even in passive aligners, or a retainer-like appliance. Different material show different property changes in similar conditions. Another study showed (Eliades 2017). Invisalign did not change significantly, although PETG material significantly increased in elastic modulus. Because Invisalign® (thermoplastic polyurethane) is constructed from two segments (hard and soft), phase separation occurs when soft segments aggregate, or line up, perpendicularly to the forces applied and breaks into smaller pieces to absorb continuing deformation (Eliades 2017, Qi 2005)

Teughels et al (2006) investigate surface roughness and the propensity for bacterial adhesion. It is known that rougher surfaces, such as crowns and denture bases show more plaque accumulation, measured in CFU, thickness, and area (Teughels 2006). As discussed earlier,

undisturbed plaque will form further rough topography which will harbor further bacterial attachment, including rods and motile organisms. This mature, rough plaque will then frequently affect the periodontium. Teughels et al (2006) measured the Ra, or surface roughness with the use of scanning electron microscopy. This is a measurement of vertical deviations of a surface from its original and ideal form. The rougher the surface, the higher the Ra (Teughels 2006). This study concludes that 0.2mm is the threshold for Ra, and once this is exceeded, biofilm formation becomes present on this surface. It is important to parallel this information with the use of aligners and consider wear of the aligner, as mentioned above in causation of these rough surfaces over wear time.

There are some roughness parameters that need to be defined. Sa (arithmetic mean deviation), Sq (root mean square root), Sz (maximum height of surface, Sv (surface volume) were used to investigate roughness in aligners specifically (Papadopoulou 2019). Indentation testing was performed to determine hardness, indentation modulus, and relaxation index using a hardness testing machine, which indented at a depth of 30 micrometers for 60 seconds on aligners samples (Papadopoulou 2019). The sample groups were a control (unused Invisalign® samples), aligners worn for one week group, and aligners worn for two weeks. Results from this study showed a significant reduction in roughness parameters in one week of orthodontic treatment and affected micromechanical retention with the attachments and tooth surface. (Papadopoulou 2019). This could be responsible for the decrease in retention of the aligner over time. The authors explain a decrease in roughness by a 'polishing' effect when the enamel surface and resin attachments make contact against the aligner material. Composites (for attachments) have a hardness of approximately 400-600N/mm^2 (Fischer 2010), and enamel surface 2866 N/mm^2, which pales in comparison to aligner material (23 times higher than Invisalign®) (Papadopoulou 2019). The discrepancy in hardness between aligners and the

surfaces it will rub on will deform or smoothen the removable appliance (Papadopoulou 2019). Lingual surfaces sit against aligner material directly, given that there are not typically lingual attachments. This big discrepancy in hardness offers insight that perhaps the lingual surfaces become more smooth than buccal surfaces. In the same study, one and two week wear both showed loss of mechanical properties when compared to the control (unworn aligner) and there is little difference between week one and week 2 aligners, proving that intraoral aging has a detrimental effect on Invisalign mechanically, in the first week. The researcher also postulates that the stresses from manufacturing of the aligners and release of plasticizers could potentially explain degradation of the aligners intraorally (Gerard Bradley 2015). It can be concluded that given the mechanical deterioration shown, two week aligner wear is not recommended.

To summarize, Alexdropolous et al (2021), found that TPU (thermoplastic polyurethane) material has higher hardness, brittleness (slight), modulus of elasticity, and less creep when compared to the the PETG (polyethylenterephthalat-glycol copolyester) products. As wear continues surface irregularities and microabrasions form inside the aligner material, which presents opportunities for bacterial accumulation (Low 2010).

Low et al (2010) observed Invisalign® samples under scanning electron microscopy (SEM) in vivo in designated time intervals of 1,3,6,12,24,48 hours and 3,7,14 days. The aligners were removed only to harvest samples. Under microscopy evaluation, samples after 7 days showed smooth crystalline structures, believed to be from calcification of biofilm (Low 2010). The texture of Invisalign® varies as it wears. The structure of unused Invisalign aligner had corrugated structure due to corresponding stereolithographic models that were used to fabricate the aligners (Low 2010). These new aligners under SEM analysis were not completely smooth; they exhibited microabrasions and irregularities (Low 2010). The distance between these peaks and valleys is 0.17mm and magnified by 845x, an undercut was present at each peak. The

undercuts measured approximately 20 micrometers. Thus, these areas were believed to be the focus of initial pellicle development and bacterial colonization (Low 2010). These surfaces provide a bacterium to be sheltered from insertion/removal shear forces of the appliance. In this study, patients were asked to clean with either water or chlorhexidine rinse. The results show that even after oral hygiene and aligner hygiene, surviving colonies remained on the aligner surface. These colonies can do one of two things; they can form pockets of planktonic bacteria if they remain on the surface long enough, or they can become loose and colonize other sites of the oral cavity with greater speed than the initial colonization (Low 2010). Low et al (2010) detected a far more complex and mature bacterial structure in areas that were sheltered and/or recessed, such as cusp tips and hollow attachment areas. Low's SEM research was believed to be the first descriptive showing Invisalign® TPU material topography under extensive magnification.

There is limited literature on transparency of aligners. Lombardo et al set out to investigate the translucency of Invisalign®, (Align Technology), F22 aligner (PD, Italy), and All-in (GE, Italy) and their effects from aging. Optical properties such as absorbance (how well color stays in the specimen) and transmittance (fraction of light that passes through the specimen) were observed from each sample by way of photospectrometry. These properties are inversely proportional (Lombardo 2015). All aligners were placed in a bath of saliva to mimic intraoral environments for two 14-day aging cycles. F22 aligners showed to have lowest absorbance, followed by Invisalign®, and All-In as last (Lombardo 2015). Although other properties are just as important, discoloration of aligners from aging is a property worth investigating further. Also, further research is needed on other properties of these chemically inert F22 aligners.

In a perfect world, thermoplastic aligners will be able to exert constant, light forces throughout the entire duration of their use. However, this is difficult to achieve (Lombardo

2016). The constant force and load from dentition causes deflection of this viscoelastic material over a period of time of wear; this is called creep (Lombardo 2016). Additionally, these forces cause deflection when the load decreases as well; this is called stress relaxation (Lombardo 2016). Four aligner materials were observed in Lombardo et al; two single layered, and two double layered. Constant load in an all-day moist, oral-like temperature-regulated environment showed a release of stress by all aligner types at 24 hours. Stress release was observed in the first eight hours, in fact. This information demonstrates that early hours of wear can show a significant stress release that could cause a significant deflection and change in behavior of the aligners. This could ultimately lead to an inaccuracy of prescribed tooth movement (Lombardo 2016).

Previously Studied Cleaning Techniques and Products

Patients in today's world are seeking esthetic solutions to tooth alignment. The result of this is continuing growth in the use of aligners for orthodontic treatment, from mild to severe malocclusions (Levrini 2016). The health of enamel and gingiva is essential to the success of an orthodontist's treatment, and thus, at home modalities must be enforced to prevent negative consequences of poor oral hygiene. However, hygienists, orthodontists, and dental assistants struggle in recommending the most effective methods of cleaning these removable appliances (Levrini 2016). It is imperative that giving correct instructions to the patients will prevent disease and assure that oral appliances remain odor-less, transparent, and an attractive orthodontic option (Levrini 2016).

Knowing that there are predispositions to biofilm formation in the oral cavity, and there are surfaces in the aligners themselves that encourage bacterial adhesion, orthodontists need to find a way to mitigate and improve these problems. We know that even after cleaning of surfaces

of removable oral appliances, planktonic bacteria may stay or can disseminate into other parts of the mouth and establish adhesion faster than the initial colonization (Low 2010). There is ample literature investigating oral appliances such as dentures and aligners in regard to best cleaning methods and agents. Currently, there has not been an established method to cleanse orthodontic aligners or retainers (Albannaa 2016). We can draw from previous studies with acrylic dentures; mainly being cleansed through mechanical brushing with a non-abrasive agents and soaking in chemical products, recommended by the American College of Prosthodontists (Felton 2011).

Albannaa et al (2016) performed an in vivo randomized clinical trial in which patients wore Essix retainers (invisacryl A Great Lakes Orthodontics, Tonawanda, NY) for 14 days and gave retainers back to the researcher. Chemical cleansing dissolving tablets were used from three brands: Retainer Brite, Kukis, and Corega. A chemical cleanser was considered to be effective if 90% of bacterial, or 500 cells were reduced (Nikawa 1999). Strict exclusion criteria was used in order to reduce potential interfering factors. Both buccal surfaces of second premolars were observed under SEM photography, and cultures of bacteria were taken and gram stained, to identify the morphology of the bacteria. Results in this study showed no statistically significant differences in bacterial count of all three chemical agents (all alkaline peroxide products). Quantifiably, there were less bacteria on the treated piece of retainer than the control. It was found that individual bacterial cells are sensitive to a disinfectant, whereas when in a biofilm they are more resistant (Albannaa 2016). This is because metabolic activity of the species is different depending if they are together with other microorganisms or alone (Peixoto 2011). Gram positive bacteria, *Streptococcus* and *Staphylococcus* species appeared to have been more disturbed by chemical agents, and that chemical cleansing after mechanical cleansing did not significantly reduce bacteria present in these Essix retainers, compared to solely mechanical cleansing (Albannaa 2016). Therefore, it was concluded that mechanically brushing the aligners

may be enough to cleanse retainers and chemical agents may be suggested to the patient if manual dexterity is a problem or if the patient is young (Albannaa 2016).

Literature shows that Lombardo et al (2017) studied the F22 TPU aligners, which were rivaled with Invisalign® and other PETG materials. F22 aligners were used to rest nine different cleansing algorithms, all a combination of ultrasonic or sonic bath, water, and either an anionic or cationic detergent. Patients were instructed to cleanse accordingly, and aligners were collected. Vibration sonic bath was at 5800 Hz, and ultrasonic bath was 42000 Hz. The maxillary first premolar was cut out, tested, and observed under SEM. It was found that five minutes of ultrasonic alone did not remove the biofilm, and the most efficacious detergent was the cationic bactericidal agent (Lombardo 2017). However, the detergent alone was not effective at removing the biofilm alone. Together, the cationic detergent and 42000Hz ultrasonic cleaner produced and statistically significant greater disturbance to the biofilms present (Lombardo 2017). The F22 TPU material, like Invisalign®, has striations in the material, making it further hydrophilic, and able to absorb water (Lombardo 2017). Ultrasonic cavitation had noticeably pitted the aligner material due to the acoustic waves and formation of microscopic bubbles. These bubbles then release enough energy when they burst to damage the bacterial cell walls and the surface of the TPU material (Trombelli 2005). This process is called ‘cavitation’ (Lombardo 2017).

Cleaning crystals are what Align Technology recommends for cleansing of Invisalign® aligners. According to the manufacturer, they are 60% sodium sulfate, 30% sodium carbonate, 7.5% sodium tripolyphosphate, 2% sodium dichloroisocyanurate, and 0.15% sodium lauryl sulfate (Shpack 2013). Shpack et al (2013) sought out to investigate the efficacy of these cleaning crystals.

Invisalign® cleaning crystals and a sonic cleaner plus Retainer Brite tablets were utilized in a study by Bernard et al (2020) to investigate the coloring effects of black tea, red wine,

coffee, and cola in Invisalign® (TPU), ClearCorrect® (TPU), and Minor Tooth Movement® (PETG). Exposure to 12 hours and 7-day exposure had similar effects in ClearCorrect® and Minor Tooth Movement® aligners, but were significantly more stained in the Invisalign® group. Black tea created the most change in spectrophotometric measurements, and the cleaning methods performed similarly, with far easier removal of black tea stains than other staining agents (Bernard 2020. Thus, Bernard suggests that Invisalign® aligners are more prone to staining than ClearCorect® and Minor Tooth Movement® from coffee and red wine. Minor Tooth Movement® aligners had a high wear resistance, strength, and dimensional stability, as well as were transparent and solvent resistant (Alexandropoulos 2021). Invisalign® aligners seemed to be more prone to pigmentation processes in both time periods studied. It has been suggested in literature that thermoplastic polyurethanes are more porous than PETG material and increasing absorption would naturally retain more pigmentation in the material (Fernandes 2013). Water molecules are attached to the aligner surface, then are internalized into the material, which then interacts with the carbamate groups in the aligner, in turn will enhance hydrophilic interactions with pigments within the aqueous environment (Liu 2016).

A more simplistic experiment was run by Levrini et al (2015), investigating cleaning agents such as sodium carbonate and sulfate crystals (Invisalign® tablets) plus toothbrushing (30 seconds), brushing alone, and running water alone (as a control) in an in-vivo environment (Levrini 2015). Scanning electron microscopy was used to analyze aligners after 2 weeks of disinfection per group. The group that performed the best was brushing combined with the cleaning tablet, followed closely by tooth brushing alone (Levrini 2015). It is evident by the achieved results that mechanical and chemical together are superior methods of cleaning aligners. From previous denture studies, brushing alone is observed to favor biofilm accumulation on dentures. Therefore, it is highly recommended to denture wearers (and perhaps

aligner wearers) that it is necessary to combine chemical action with mechanical action (Paranhos 2007). De Andreade et al (2010) found that effervescent tablets were superior to an ultrasonic device at removing *streptococcus mutans*; although were not as good at removing *candida albicans*. All tested aligners in research by Levrini et al displayed remaining organic microbiological contamination and some with inorganic crystalline structures (Levrini 2015). The contamination remaining was of spherical-shaped bacteria, often not seen as individual colonies, because they grow into one or multiple layers and become covered by an amorphous substance (Levrini 2015). Kiesow et al. studied efficacy of denture cleaning tablets, toothpaste, mouthwash, isopropyl alcohol, household bleach, vinegar, and soap on denture material, PMMA (polymethyl methacrylate). It was shown that some products, such as bleach, mouthwash, and isopsopyl alcohol were destructive to the surface of the PMMA, causing loss of gloss, scratching, and pitting. Vinegar and soap trials were shown to be compatible with the PMMA material, however, was not effective microbially. The conclusions suggest that denture cleansing tablets provide the best mixture of material compatibility and microbially effectiveness (Kiesow 2016).

Chlorhexidine studies as previously discussed were important to analyze plaque adhesion levels and efficacy. Ribiero et al (2007) conducted a systematic review of formularies of chlorhexidine (gel, varnish, and solutions) and the effect those have on reduction of *streptococcus mutans* in the oral environment. Researchers found that chlorhexidine mouthwash had only short-term effects on *streptococcus mutans* in saliva (Ribiero 2007). Intensive and frequent application of 1% chlorhexidine gel and 1% chlorhexidine varnish showed exceptional effects on *streptococcus mutans* lasting 4-26 weeks (43). 40% chlorhexidine formulary provides more of an effect on the period of lowered MS than the 1% chlorhexidine formulary (Ribiero 2007). Perhaps this intensive treatment with chlorhexidine gel and varnish offers orthodontists another option for intraoral reduction of pathogenic bacteria.

Propolis, also known as ‘bee glue’, is a naturally occurring mixture of resin-like and balsam-like material that bees acquire from flowers and trees and integrate with their salivary secretions and beeswax (Sugio 2020). The word is derived from ‘pro’ (in front of), and ‘polis’, (community), meaning that this is a defense for hives (Fokt 2010). Bees use this resin-like sap to build hives. Composition of propolis is 50% resin, 30% beeswax, 10% essential oils, 5% pollen, and 5% other organic substances (Hwu 2014). Propolis is known to have anti-bacterial, anti-inflammatory and immunoregulatory properties. Propolis can affect the cell’s cytoplasmic membrane, prevent motility of bacteria and cell adhesion, and inhibit formation of water insoluble glucan (Loesche 1986). Apigenin and tt-farnesol are chemical groups present on the structure of propolis, which are important inhibitors of glucosyltransferases (GTFs) (Loesche 1986). This creates an inability for glucans to be synthesized, and thus inability for the bacteria to bind to a pellicle (Mohsin 2015). Flavonoids, found in propolis, are other well-known to have antibacterial activity. These bacteriostatic properties affect a plethora of bacterial species and in high concentrations, propolis can also be bactericidal (Liberio 2009). These properties may be observed in an acidic pH and at higher temperatures (37 deg Celsius). Ethanol extract of propolis (EEP) was specifically found to be effective against *streptococcus mutans,* showing its bacteriostatic and bactericidal effects in an acidic pH, followed by a neutral environment, and then an alkaline environment pH5 and higher (Yang 2020, Mohsin 2015). The effects were seen to be greatest on gram positive bacteria and yeasts, and less effective on gram negative bacteria (Hwu 2014). Anti-inflammatory properties by propolis can be attributed to its ability to inhibit prostaglandin synthesis, promote immunity of the cell and result in healing in the epithelium (Liberio 2009). Collagen formation is also promoted by propolis, resulting from containing iron and zinc (Liberio 2009). In dentistry, it has been studied in prevention of dental caries and

biofilm inhibition on tooth surfaces and removable dental appliances (de Souza 2019). In extraoral uses, it is used to help skin heal.

For its antifungal properties, propolis was tested in research in prevention of denture stomatitis, in lieu of other more hepatotoxic antifungal agents such as amphotericin B, nystatin, and miconazole (Sugio 2020). Meta-analysis by Hwu et al (2014) showed that treatment with propolis compared to gels, mouthwashes, and placebos, propolis decreased the quantification of candida and *streptococcus mutans* counts intraorally and decreased clinical signs of denture stomatitis. Effectiveness was observed with use of propolis and was deemed comparable to other remedies for denture stomatitis such as nystatin and chlorhexidine (Sugio 2020). De Souza et al (2019) showed propolis having an immediate effect on microbes *streptococcus mutans* and *canada albicans,* however a more delayed effect on denture biofilm formation. The recommendation was to apply commercially available natural cleaning agents containing propolis; some notable brand names are Propolinse, Bee Healthy Farms, and Toms of Maine toothpaste and Probee (De Souza 2019).

Propolis toothpaste was utilized in young children aged 7-12 years old in research by Mohsin et al (2015). Three minute daily use was encouraged for four weeks and salivary samples were collected at the end of the fourth week. Quantification was tested by Dentocult SM strep mutans Kit (Oy, Finland). *Streptococcus mutans* counts at week one and four were observed to have a significant reduction ($p<0.0001$) when compared to salivary samples collected before propolis use (Mohsin 2015). In-vivo, this dentifrice showed a benefit to prevention of dental caries in young patients. This same age group is who orthodontists treat, as opposed to previously discussed denture-wearing population.

Nanosilver was also implicated in its antibacterial activity intraorally. Toothpastes may contain two chemical agents that assist in such antimicrobial activity: chitosan and nanosilver

particles. Chitosan is typically found in chitin, or insect exoskeleton and Nanosilver is formulation of deionized H20 and particles of silver in a suspension (Ahmed 2019). Three toothpaste formulations were tested in this study: nanosilver, chitosan, and fluoride toothpaste, with a control. Zones of inhibition were examined on salivarius mitis agar plates for each toothpaste formulation. With a far larger zone of inhibition of 20.14mm (mean) observed, Group 1, nanosilver, was the highest performing group, followed by fluoride (16.01mm mean), and chitosan (10.84mm mean) performing the worst (Ahmed 2019). Silver nanoparticles were discussed to have produced pits, or outer cell membrane surface changes due to the continuous lipopolysaccharides and membrane proteins (Amro 2000). One may consider the ingestion of these silver nanoparticles to be potentially harmful to the gastrointestinal tract and potentially cause inflammation. Authors argue that a toothpaste is not meant for ingestion, as it is for topical use only (Gaillet 2015). Second to the nanosilver performance, 1000ppm fluoride toothpaste was effective against *streptococcus mutans* (Ahmed 2019). Fluoride is known to prevent formation and progression of carious processes, which is very well documented in literature. Although, at high concentrations of bacteria is too high, fluoride is unable to overcome the bacterial challenge (Featherstone 2004). Dos Santos et al (2014) combined Nanosilver, chitosan, and fluoride in a formulary that consisted of nanosilver fluoride (NSF) and showed that NSF is effective at arresting caries without causing tooth staining. Researchers nicknamed NSF the ‘silver bullet’. This study was conducted as a randomized clinical trial as H20 drops and NSF drops were randomly given to subjects. NSF drops were in contact with tooth surfaces for two minutes, each tooth getting approximately 10mg of the solution (dos Santos 2014). 60 school children with 130 decayed primary teeth participated in this study. After seven days, 81% of decayed teeth in the NSF group were shown to be arrested, and after five months, 72% of teeth showed complete arresting of decayed teeth, where this only occurred in 27% of the control group (dos Santos

2014). During the arresting process, the lesions did not turn black, whereas in the control group, arrested caries did turn black. This was deemed to be a stigmatizing and negative association to children being perceived as poor. Compared to silver diamine fluoride (SDF), currently on the market, NSF does not have a metallic flavor, does not oxidize when in contact with oxygen, and is relatively inexpensive (dos Santos 2014). These benefits are important to note, in that NSF may become a worldwide solution for childhood caries in communities of poverty (dos Santos 2014). This study is important to draw from in the formulation of a product that can essentially stop carious processes. With removable aligner wear, the concern for orthodontists is that carious processes would not only continue but would be started because of poor oral hygiene while in removable appliance treatment. Perhaps the future of antimicrobial aligners would include a protective chemical layer within the aligner containing NSF.

Copper containing solutions have also made a breakthrough in research in cleansing clear aligners. Cupral is a compound containing copper, calcium, and hydroxide, available in a paste and has been shown to have antimicrobial activity, and currently used in periodontal and endodontic infections (Meto 2019). Meto et al (2019) investigated effects of aligners that were exposed to cupral vs not exposed in a 2019 case report. Transmitted-illumination confocal microscopy analysis, or 3d mapping, was used to compare samples before and after exposure. Microbiological assessment of aerobic and anaerobic microorganisms were evaluated. Cupral treated aligners showed non-detectable colonies both in the aerobic and anaerobic conditions (Meto 2019). Because this study was observing all bacterial species, discussion on summer vs winter variations were important. Seasonal climate affects the species that one would find, as the winter season brings more upper respiratory tract disease in which *S. aureus, H. parainfluenzae,* and *N. mucosa* (Meto 2019). These were found more in the January through March sampling because upper respiratory tract species are also found in the oral cavity during this time of year.

In summer, *streptococcus mitis* and *streptococcus oralis* are most detectable in the aligner appliance. Potent effects were found from the Cupral compound against the naturally occurring biofilm structure present on orthodontic aligners. While expanding research on Cupral is needed, it could become a novel tool for removable thermoplastic aligners (Meto 2019).

Gold Nanoparticles were used by Zhang et al (2020) to test efficacy against *P. gingivalis* in orthodontic aligners. Arguments on biocompatibility exist for any nanoparticles that may be used intraorally. Gold nanoparticles were proven to be biocompatible as AuNP which is chemically stable and size controllable, and were combined with DAPT (4,6diamino-e-pyrimidinethiol) (Zhang 2020). Conjugated molecules show effects against gram negative bacteria and have little influence on drug resistance than conventional antibiotics. *Porphyromonas gingivalis* is a gram negative anaerobic known pathogen in causing periodontitis and has been identified in diseases in the body such as atherosclerotic vascular disease, chronic nephritis, and diabetes mellitus. (Teratani 2012, Holmlund 2017, Graziani 2017, Liljestrand 2018). This suggests that *P. gingivali*s can be transient in the bloodstream while flossing or brushing and chewing, causing it to be found in the coronary arteries, liver, and brain. (Zhang 2020). Clear aligners stay on teeth nearly all day. This study suggests that placing a AuDAPT coating on the inside of Invisalign aligners will not only keep gram negative bacteria at bay, but assist in decreasing the quantity of planktonic cells, and prevent biofilm formation. SEM and Confocal Laser Scanning Microscopy were used to view surfaces of aligners with and without AuDAPT coating and results were astounding (Zhang 2020). On AuDAPT coated aligners, no bacterial cells were found after initial immersion in 10^4 CFU/mL of live bacteria. (Zhang 2020) Biological safety was performed on umbilical vein endothelial cells after exposure to AuDAPT and it was proven that it does not affect viability of these cells in >100microgram/mL concentration (Zheng 2017, Zhang 2020) Mucosal irritation was conducted with AuDAPT, in

which microscopic evaluation was conducted looking at long-term contact and gingival irritation. No mucosal irritation was found in tissues which contacted the compound. For this reason, AuDAPT may have oral applications (Zhang 2020) and perhaps there is a future for antibacterial coating of aligners.

Perhaps a more radical approach, ozone has been shown to inhibit microbial growth. Murakami et al used ozone to disinfect dentures in 1996. Ozone is claimed to have high biological safeness, is a strong disinfectant, and deodorizer (Murakami 1996); giving it a potential use in denture cleansing. Murakami et al (1996) sought out to see if ozone was a viable option for denture cleansing and effectiveness against candida albicans. An ionizer and ozone-concentration measuring device were used to accomplish this. Sample was placed into a tank for 72 hours at 25degC with an ozone concentration of 10ppm, and later plated. Initial C albicans decreased by 1/10^3in 60 minutes, although complete elimination of the bacterium was not possible. Their argument comes from a statistic from the USA that 150 cases of misuse and accidental ingestion of denture cleaning agents are reported per year (Murakami 1996). The researchers concluded that further research is needed on this topic, but ozone has strong benefits in disinfection and deodorizing dentures, its short half-life in water and quick decomposition makes it a suitable cleaning agent (Murakami 1996). Perhaps ozone could be a cleansing agent with clear aligners as well. Further research is needed to explore this cleansing method.

Numerous cleaning agents and methods have been explored to clean clear aligners and other removable oral devices such as dentures and partial dentures. Products discussed include Invisalign cleaning crystals, effervescent clear aligner calcium hydroxide tablets, ultrasonic devices, sonic bath, nanosilver particles, chitosan, cupral, chlorhexidine, and more natural agents including propolis and ozone. Methods include rinsing, brushing with a dentifrice, vibration, and

chemical only agents. It is important to keep these methodologies in mind when designing an experiment to further assess the effectiveness of these agents.

Quantification Methods Previously Used

Literature on the efficacy of cleaning of orthodontic aligners is potent with information, however differences in the way this information was quantified and qualified exist. Scanning electron microscopy (SEM) is by far the most used. The samples must be completely dry before undergoing microscopy. Aligner observation by Lombardo et al (2017) included focusing the scanning electron microscope and sample enlarged by ten thousand times and saved with an 8-bit color depth grey scale. In this research, SEM was utilized to determine the best method of cleaning F22 (TPU) orthodontic aligners was 5 minutes of ultrasound treatment (42kHz) along with 0.3% cationic germicidal benzalkonium chloride (Lombardo 2017). Levrini et al (2015) placed aligner cutouts from cusp tips and interdental papilla under SEM analysis. SEM stubs are coated with carbon-based adhesive, gold coat and run in an argon environment at pressure of 1x10^-1 mbar (Levrini 2015). Acceleration voltage used to visualize all samples was set to 7kV, producing images that are 8bpp grayscale and in identical pixels (Levrini 2015). Surface details of the aligners with heavy biofilms are often cracked in appearance due to the desiccation process required prior to SEM observation. This can assist in discerning the thickness of the colonies. In this research, SEM helped to discover that on aligner material, thick biofilms appear as if they are peeling in samples that were used as control under 50x magnification, indicating the severity of the biofilm.

Low et al (2010) was the first to assess Invisalign thermoplastic polyurethane and visualize under SEM low magnification the corrugated intaglio surface and microabrasions on smooth surfaces. Under 845x magnification, researchers were able to describe the peaks of the

corrugated surface to be 0.17mm, and visualize an undercut area of approximately 20 micrometers, which would provide a perfect environment for organisms to accumulate. Low et al (2010) visualized that at 24 hours, the cusp indentation of the thermoplastic aligner was heavily populated with fast plaque groups in mostly cocci, spherical shape and covered in thick EPS (extracellular polysaccharides). Later in observation of aligner materials with SEM at 14 days, smooth crystalline structures, or calcifications for biofilms were observed (Low 2010). It was discussed that dehydration protocols required prior to SEM observation, could potentially introduce shrinkage, deformation, and distort the biofilm from its original configuration (Fischer 1988). Fisher et al describe the extracellular matrix, because of its aqueous nature, is susceptible to distortion by dehydration up to one percent of its original volume (Fischer 1988). This SEM technology excels in qualitative visualization of morphological characteristics of bacteria on aligner surfaces. However, there has been no technology that has been invented with SEM that can interpret quantitative information about these surfaces, which is a drawback to SEM. Eliminating the need for dehydration could reduce future sample distortion (Slayter 1992). Nonetheless, Low et al (2010) were able to describe the surface of aligner to be irregular with microabrasions using SEM, and this itself is an advancement to our understanding of bacterial adhesion in orthodontic aligners.

Scintillation method of quantification was used by Steinberg et al. with *streptococcus sobrinus*. Bacteria were radioactive labelled and tested on several orthodontic appliances such as coil springs, edgewise brackets, elastics, and modules, each showing different adsorption. KCl wash was placed on all samples and then placed in a scintillation fluid of 10mL and counted with a scintillation counter (Steinberg 2004). The scintillation counter reads radioactive counts per minutes in each millimeter squared. From this data using scintillation, it was shown that modules

were the most susceptible to *streptococcus sobrinus* in adherence to an existing pellicle, with springs being the least (Stainberg 2004).

Colorimetry is a technique that is used to determine the concentration of colored compounds in a material or solution (Bernard 2020). Included in this is luminosity, which ranges from dark to light (0 being dark; 100 being light), and two values (a and b) from a chromatic scale (positive a being red; negative a being green, and positive b meaning yellow and negative b meaning blue) (Bernard 2020). 300 aligners were scanned in four time intervals of use to obtain color parameters and assess color change of orthodontic aligners after submersion in several potentially staining agents. This method of observation of stains helped to conclude that thermoplastic polyurethane (Invisalign®) has a more porous composition, allowing more penetration of color (Bernard 2020). It also helped conclude that coffee and red wine stained aligners the most, compared to black tea, cola, and saliva (Bernard 2020).

ATP bioluminescence was used in two literatures cited. This method is utilized for years for monitoring surface hygiene, or cleanliness in the food industry (Davidson 1999). In this setting, hazards such as contamination of surfaces that food comes in contact with (Davidson 1999). These methods were relatively simple to use and could deliver quick quantitative and qualitative results. It is a rapid testing tool, which is necessary in this setting, as opposed to hygiene swabbing techniques which would take several days to evaluate the cleanliness of surfaces. Davidson et al (1999) sought out to determine if ATP bioluminescence was a purposeful way to analyze these surfaces. *Escherichia coli* were purposefully inoculated on a food-grade stainless steel counter and were tested using ATP bioluminescence and swabbing. The test used was Biotrace Clean-Trace Rapid Cleanliness Test. Testing with this includes a swab with dacron bud, a cationic agent (premoistened) to release ATP from microbial or food cells and to assist in remove this from surfaces (Davidson 1999). A reagent is used which reacts

with ATP on the swab and the light intensity is measured-- as intensity of the light increases, more ATP has collected, which reads as a higher degree of contamination. Although ATP bioluminescence was able to determine a minimum detection limit of contaminants, hygiene swabbing was superior at identifying this contamination. The authors concluded that in a real-life scenario, where *E. coli* is not the only contaminant, and in far less concentrations, there may be a lesser performance of just ATP bioluminescence itself.

The bioluminometer units are units of relative light. Orthodontic aligners were observed with bioluminescence after different cleaning methods by Levrini et al (2016). Cleaning methods were water alone, brushing with toothpaste, and brushing with toothpaste plus a sodium carbonate tablet (Levrini 2016). Highest bacterial concentration was found on cleaning with water solely, followed by brushing with toothpaste only, and brushing with toothpaste plus a cleaning crystal produced the lowest relative light units. A Bioluminometer System Sure II Plus (Parma, Italy) was used with a SuperSnap kit moving from molar to molar aligners of 20 participants who utilized the previously mentioned cleaning methods. These two articles suggest that bioluminescence was certainly a viable and reliable tool of analyzing preliminary bacterial colonization on surfaces, whether it be from food preparation to thermoplastic aligners (Levrini 2016, Davidson 1999).

Lombardo et al (2015) showed that certain agents had more staining potential with the use of spectrophotometry. Comparing F22, Invisalign®, and All-In® aligners, researchers analyzed the absorbance and transmittance to these aligner materials after being subjected to two cycles of 14-day submersion in an oral-like environment. Two wavelengths of light were used (400 nm and 700 nm) and it was determined that F22 aligners had lowest absorbance, and highest light transmission; ultimately outperforming Invisalign and All-In after both aging cycles (Lombardo 2015). These optical properties simulated the oral environment, as artificial saliva

was used as the medium in-vitro. The researchers suggest that chemicals, such as sports drinks, and mechanical stress, such as bruxism, were not delivered in this simulation which would have not simulated fully what may happen to the optical performance in an in-vivo environment (Lombardo 2015). However, spectrophotometry was able to assist in determining optical properties of three different aligner brands.

As per literature on any microbiology, the gold standard is counting colony forming units (CFU) on agar plates (Stingl 2021). There are some advantages that come with this. This method provides the capacity to produce dilutions, where colonies may be counted directly. CFU method also provides the ability to only count the thriving and live bacteria- it does not allow debris and dead cells to be counted (Stingl 2021). A disadvantage is that colonies clump together, and sometimes are not able to be discerned, leading to miscounting. CFU method is tedious and time consuming, and much be examined at specific time points for optimal and reliable data (Stingl 2021). An expedited alternative method to counting colonies is using the Denocult SM Reader. Specifically testing presence and levels *of Streptococcus mutans*, this kit is acclaimed to be a paramount test (Deepti 2008, El Nadeef 1992, Mohsin 2015). The test has high statistical significance and reliability. It can be done chairside, is not time-consuming, and requires basic armamentarium. Sensitivity and specificity are shown to be better than conventional methods (Karjailainen 2004). Mohsin et al (2015) used the Dentocult SM reader to test efficacy of propolis as an antimicrobial agent for school aged children. Much thought has been placed on the potential for using the Dentocult SM reader vs plating bacteria.

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STATEMENT OF THESIS

The aim of this study is to quantify colonies of *streptococcus mutans* present on thermoplastic aligners after they have been cleaned with products readily available to patients. These products are widely recommended to patients as cleaners by orthodontists; therefore, the purpose of this investigation is to see if they are successful at removing *streptococcus mutans* from a popular thermoplastic aligner material, DX30. The thermoplastic material DX30 (Trutain, Rochester, MN) was selected because it is a low-cost material that has been popular since 1975. A secondary purpose is to shed light on the longevity of effectiveness of cleaning agents on thermoplastic plastic using optical density.

MANUSCRIPT FOR PUBLICATION

The following paper was prepared according to the guidelines published in the American Journal of Orthodontics and Dentofacial Orthopedics.

EFFICACY OF OVER THE COUNTER PRODUCTS IN REMOVAL OF *STREPTOCOCCUS MUTANS* FROM THERMOPLASTIC ALIGNERS – AN IN VITRO STUDY

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ABSTRACT

Background and Significance: The market for clear aligner therapy has been steadily expanding. Thermoplastic materials are commonly used to fabricate both clear aligners and retainers. Since the mouth is a septic environment, these aligners need to be cleaned periodically.

Purpose: The purpose of this study was to evaluate the effectiveness of five disinfectant methods commonly recommended to patients by their orthodontist to clean thermoplastic materials.

Methods: Molar segments cut from a popular commercially available thermoplastic aligners (Trutain, Rochester, MN, USA) were inoculated with *streptococcus mutans* and incubated for 48 hours in brain- heart infusion media to establish a biofilm on the thermoplastic segment. Segments were rinsed for 10 seconds with distilled water, dried and then cleaned with one of the following products. A commercially available foam product (WhiteFoam, Inglewood, CA, USA), a commercially available effervescent tablet (Retainer Brite, Centerbrook, CT, USA), dish soap (Dawn Dish Soap, P&G, West Virginia, USA) and toothbrush, white vinegar, or mouthwash (Listerine, J&J, New Jersey, USA), all according to manufacturer's directions. Following these cleaning procedures, a sample was taken from each aligner segment and plated on blood agar. Colony forming units were counted at 24 hours, and optical density was recorded at 24 hours and 48 hours.

Results: Agar plates with thermoplastic segments treated with foam, effervescent tablets, or mouthwash did not show presence of *streptococcus mutans* at 24 or 48 hours. Dish soap with toothbrush and vinegar treatment showed significant *streptococcus mutans* colonies at 24 hours and 48 hours. Optical density readings at 24 hours and 48 hours showed continued increase in *streptococcus mutans* colonies for samples treated with dish soap with toothbrush, and vinegar, whereas samples treated with foam, tablets, or mouthwash showed no evidence of *streptococcus mutans* at 24 hours or 48 hours.

Conclusions: Antibacterial treatments were more effective than mechanical methods or vinegar for removal of *streptococcus mutans* from thermoplastic molar segments.

INTRODUCTION

According to a national survey by YouGov, 45% of adults are dissatisfied with their smile, and 20% of adults would like to undergo orthodontic treatment to correct esthetics[1]. Thus the demand for esthetically acceptable methods for aligning teeth is strong. The use of clear thermoplastic aligners to move teeth was first introduced by Jack Sheridan[2] and subsequently commercialized using CAD/CAM technology by Invisalign[3]. This new therapy was esthetically desirable. The ease of use and the almost invisible appearance of the clear aligners revolutionized the orthodontic market. Recently there has been an increase in the use of in-house custom fabricated thermoplastic aligners. Thermoplastic aligners and retainer may be fabricated from polyurethane, polyester, and polypropylene[4]. Regardless of the thermoplastic material used, the mouth is a septic environment and any material put into this environment is quickly coated by a complex biofilm containing both salivary proteins and pathologic bacteria. *Streptococcus*

mutans is the predominant gram-positive aerobic bacteria that causes caries and white spot lesions. Individuals using thermoplastic aligners are at similar risk to those with fixed orthodontic appliances for dental caries and decalcification[5,6]. Both demineralization and dental caries can be prevented by disrupting this biofilm. Therefore, it is important to disinfect removable aligners and providers recommend a variety of cleaning options based on their clinical experience. This results in the use of many different recommendations from dental professionals regarding cleaning methods. Although commercial manufacturers have stated that their products are effective, there is minimal independent scientific evidence to support those claims. Therefore, practitioners are forced to rely on commercial product inserts and/or clinical experience when making recommendations.

One thermoplastic material that has been popular since 1975 (DX30, Trutain) is still being used today due to its availability, low cost, and reputation. Commercially available products currently recommended for aligner cleaning include effervescent tablets, antibacterial foams, dish soap and toothbrush, mouthwash, vinegar, and ultrasonic baths are often recommended. The aim of this study is to evaluate the efficacy of some of these methods in reducing *streptococcus mutans* colonization on thermoplastic aligners made from DX30.

METHODS

All equipment was used following the CWRU laboratory protocols and guidelines, including general biosafety standards. Eighteen molar segments were fabricated from 0.03 thick, 125mm circular thermoplastic sheets (DX30-25 (Trutain). A pressure-molding instrument (Biostar, Scheu-Dental, Germany) was utilized and thermoplastic aligners were formed to specific material heating and cooling parameters. Following formation, molar segments on teeth with buccal attachments were cut out and molar segments were placed in a sterile cell culture

dish (Thermo-Fisher Scientific) and were sterilized for 30 minutes under UV light (Spectrolinker, XL 1000). The thermoplastic material used for this experiment was an unlinked polyurethane (DX-30-25, Trutain). *Streptococcus mutans*, a facultative anaerobic gram-positive coccus, in the form of KwikStik ATTC 25175 (Microbiologics®) were grown in BHI (Brain Heart Infusion) media used to inoculate each molar segment.

Prior to inoculation of the sample, purity controls were tested for BHI, bacteria, and BHI + bacteria. Inoculation was performed in cell culture dishes, which were then incubated at 37°C for 48 hours to maximize biofilm formation. The presence of a biofilm was confirmed under 4x magnification with a laboratory microscope prior to testing cleaning techniques.

Controls: (N=6) Controls were each rinsed under distilled water for 10 seconds. A sterile one microliter loop was used to swab the aligner segment in the orthodontic resin attachment and cusp tips, and was streaked on a 5% sheep blood agar plate.

Foam: (WhiteFoam, Inglewood, CA, USA) molar segments (N=6) tested using a 10 second rinse under distilled water. As per manufacturer's direction, foam was applied on the intaglio and allowed to sit for 5 minutes prior to swabbing and streaking on a blood agar plate.

Effervescent Tablets: (Retainer Brite, Rochester, MN, USA) molar segments (N=6) tested using a 10 second rinse under distilled water. As per DFU, one tablet was suspended in warm water, aligner segments were dropped into this suspension for a total of 15 minutes prior to a secondary 10 second rinse under distilled water. Segments were swabbed and streaked on a blood agar plate.

Dish Soap (P&G, West Virginia, USA) molar segments (N=6) were rinsed under distilled water for 10 seconds. A new toothbrush was dipped in dawn dish soap and aligner pieces were

scrubbed for 10 seconds, followed by an additional 10 second rinse under distilled water. Segments were swabbed and streaked on a blood agar plate.

Vinegar molar segments (N=6) were rinsed for 10 seconds under distilled water, and dropped in a suspension of half-warm water, half-white vinegar for 15 minutes prior to an additional 10 second rinse under distilled water. Segments were then swabbed and streaked on a blood agar plate.

Mouthwash (Listerine, J&J, New Jersey, USA) molar segments (N=6) were rinsed for 10 seconds under distilled water and placed in a Listerine solution for 15 minutes prior to a secondary 10 second rinse under distilled water. Segments were swabbed and streaked on a blood agar plate.

After cleansing, each aligner segment was placed in a secondary marked cell culture dish filled with 15mL pure BHI broth. All cell culture dishes were incubated at 37°C for 48 hours, with optical density analysis at 24 hours and 48 hours. Blood agar plates were analyzed at 24 hours and 48 hours. Colonies were visible and able to be counted at 24 hours. They were enumerated manually and photographed at 24 hours and 48 hours. A 100mL sample was taken from each cell culture dish, which contained 15mL of broth and the cleaned aligner segment, at 24 hours and 48 hours. The samples were observed at each time point in a spectrophotometer (Biotek Synergy 2) at 600nm wavelength.

RESULTS

Biofilm accumulation was visible at 48 hours after incubation and was most clearly observed at cusp tips and attachment wells. Bacterial colonies (CFU) were visible and could be counted 24 hours after incubation *(Table I).* However, because the colonies had multiplied and

coalesced they were too dense to be accurately counted at 48 hours. This was expected because *streptococcus mutans* double in population within 1.1 hours resulting in a greater than 1,000-fold increase between hour 2 and 12 hours[7]. Optical density readings taken 24 hours after cleaning showed bacteria remained on aligners that were cleaned with dish soap/toothbrush, and vinegar. The other methods did not show any bacterial growth at 24 or 48 hours.

Table I: Mean and SD of bacterial plate counts and OD readings 24h, 48h

	Bacterial Counts		**Optical Density Read**	
	mean	**SD**	**24h**	**48h**
Control	>300	0	0.906	1.157
Foam	0	0	0.142	0.139
Tablet	0	0	0.156	0.15
Dish Soap	117.5	113.52	0.821	1.344
Vinegar	232	126.42	0.822	1.81
Mouthwash	0	0	0.156	0.251
BHI Only	0	0	0.129 (BHI media)	0.136 (BHI media)

Table I: *shows that foam, tablet, and mouthwash were successful in removal of s. mutans and dish soap with toothbrush, and vinegar cleansing were not. Density read suggests similar results as the bacterial counts, with additional information on longevity of product success for foam, tablet, and mouthwash.*

DISCUSSION

While using more esthetic approaches to moving and retaining teeth, proper maintenance and cleaning procedures should be used to prevent periodontal disease and caries. The methods of cleaning used in this study were chosen due to popularity, classification, and availability of the product. There is limited current research testing these types of products on thermoplastic materials. This study is the first to provide information on lasting effects of cleaning products.

Products that are advertised as antimicrobial include foam, tablets, and mouthwash; these are all chemical agents and they performed the best. In contrast, dawn dish soap is not antimicrobial. It is a surface-active agent that lowers surface tension within the medium as well as the interfacial surface tension among two liquids and creates micelles[8]. There are many

environmental applications of a surfactant, including removing unwanted metals or inorganic compounds from soil. In this investigation, samples cleaned with dish soap in conjunction with a toothbrush appeared visibly clean at cusp tips and attachment wells. However, significant bacteria remained on the aligners. It is possible that higher water rinse temperatures and longer cleaning times might have been needed to kill S. mutans colonies[9]. Such temperatures are not practical for thermoplastics as they induce deformation of the material.

Vinegar has acetic acid as its main active ingredient (5%) leading to a low pH of 3.1. Vinegar has been shown to help remove alkaline stains, grime from hard water, and mineral buildup, which are all alkaline[10]. Agarwala et al. found that vinegar reduces polyurethane flexural modulus, making a thermoplastic retainer less flexible due to underlying chemical changes such as swelling and degradation[11]. Although vinegar has been shown to have antimicrobial properties, perhaps the surface of aligners does not contain enough alkaline material to remove and may have detrimental effects on the plastic itself.

Listerine is a mouthwash with a unique patented formula containing essential oils (eucalyptol, menthol, and thymol) which have been shown to reduce plaque levels and inhibit anaerobic bacterial species[12]. Because mouthwash is not a clear liquid, there is the potential for staining of aligners due to pigment adsorption of thermoplastic material.[13] This is probably not a major concern for aligners that are changed every two weeks but is a possible area for further research if mouthwash is used over an extended period such as post orthodontic retention. Staining is not an issue if retainers are only worn at night but full-time wear might pose a problem.

Mechanical interventions such as brushing with toothpaste have been shown to introduce scratches to the surface of the plastic, therefore decreasing light transmittance, and causing a

milky white appearance of the plastic. A study by Fine et al. showed that Listerine had only a minor effect on light transmittance after a 6-month cleaning regimen[12].

Cusp tips were observed to have the most biofilm growth (fig 1a) in this study. This finding was consistent with a SEM (scanning electron microscopy) study published by Low et al [14.]. They observed a more "mature and complex biofilm formation" in protected areas of aligners such as cusp tips and hollow attachment areas. All three of the methods that were effective were able to remove this biofilm and the embedded S. mutans in our study.

Cost is a consideration when making a recommendation to orthodontic patients. If WhiteFoam (Inglewood, CA, USA), Retainer Brite (Centerbrook, CT, USA), and Listerine (J&J, New Jersey, USA) products are all effective at removal of S. mutans from thermoplastic materials, the lowest priced category of cleaning product should be recommended *(Table II)*. Price per use for WhiteFoam and Retainer Brite tablets were both $0.17 per use and Listerine, given a 40mL minimum for submerging the aligner entirely, was shown to cost $0.23 per use. However, if a household is already using mouthwash it might be acceptable to use for aligner cleaning as well as "fresh breath".

Up to now, orthodontists have been giving cleaning recommendations to patients based on anecdotal evidence or manufacturers claims. This study aimed to provide insight into methods that work to remove microbial biofilm from thermoplastic materials. Although dish soap and vinegar may have been previously recommended by orthodontic specialists, it is evident that chemical antimicrobial agents such as foam, effervescent tablets, and mouthwash are more effective in killing *streptococcus mutans* that grow on thermoplastic aligners and retainers.

A limitation of this study was that only one thermoplastic material was used, and only one product in each product category was tested. Further research is needed to investigate the

color stability after long term use of a colored mouthwash such as Listerine (J&J, New Jersey, USA) and the efficacy of these treatment on different aligner materials.

Table II: Cost of Products Tested (price per use)				
White Foam	**Retainer Brite**	**Dawn Dish Soap**	**White Vinegar**	**Listerine**
$19.99 /1/50mL	19.99/ 120tab	$2.99 19.4floz	$1.98 64floz	$5.69 1L
$ 0.17 per use	$0.17 per use			$0.23 per use

***Table II**: Product cost per use.*

CONCLUSIONS

- Foam, tablets, and mouthwash successfully remove *streptococcus mutans* from thermoplastic aligners made from DX30-25 (Trutain, Rochester, MN, USA).
- Contrary to popular belief, dish soap/toothbrush and vinegar soak were not effective for removal of *streptococcus mutans* from thermoplastic aligner DX30-25 (Trutain, Rochester, MN, USA).
- Chemical methods of cleaning are superior to mechanical methods.
- Mouthwash was the most convenient effective product but Retainer Brite and Foam tablets were most cost-effective.

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APPENDICES

MATERIALS & METHODS

1. Materials

1.1 Required machinery:

Required laboratory equipment that was already present at CWRU dental school microbiology department included refrigerator and Bunsen burner. Materials purchased for this project included a microbiological incubator and an autoclave. All equipment was used following the CWRU laboratory protocols and guidelines, including general biosafety standards. All surfaces where the experiment was performed underwent disinfection prior and after laboratory procedures.

1.2 Other materials used:

- Brain Heart Infusion broth (Remel R452472)
- Blood Agar Plates; 5% sheep blood (Thermo-Fisher Scientific™)
- Sterile pre-packaged single-use inoculation loops 1microliter* (Fisherbrand ^R)
- Sterile micropipette tips (Sharp™, Deville) *?
- Cell culture plate (Thermo- Fisher Scientific™ sterilin™)*
- KwikStik ATCC 25175 (Microbiologics®)
- CO2 Gas Generating pouch system (BD GasPak EZ™)

2. Methods

Eighteen molar segments were fabricated from 0.03 thick, 125mm circular thermoplastic sheets (DX30-25, Trutain) A pressure-molding instrument from (Biostar ,Scheu-Dental, Germany) was utilized and thermoplastic aligners were formed according to specific material heating and cooling parameters . Following formation, molar segments on teeth with buccal attachments were cut out; approximately four molar pieces per dental arch. After the thermoforming process and aligner sectioning, each plastic molar segment was placed in a sterile cell culture dish (Thermo-Fisher Scientific) and put under a UV light (Spectrolinker, XL 1000) for 30 min on each side. The thermoplastic material used for this experiment was an unlinked polyurethane (DX30-25, Trutain).

Bacterial Strain

Streptococcus mutans is a facultatively anaerobic gram-positive coccus. The KwikStik ATCC 25175 (Microbiologics®), stored at 2-8 °C individually packed in a foil pouch and grown by direct (broth) method. The ampule (KwikStik) was broken to release hydrating fluid to hydrate the microorganism pellet. The pellet was crushed until the suspension was homologous and the inoculation stick was then placed in one milliliter liquid medium (Brain Heart Infusion, BHI). The tube was placed inside the incubator for 24hs at 37°C. Characterization of *s. mutans* colonies are small and white *(Figure 1).*

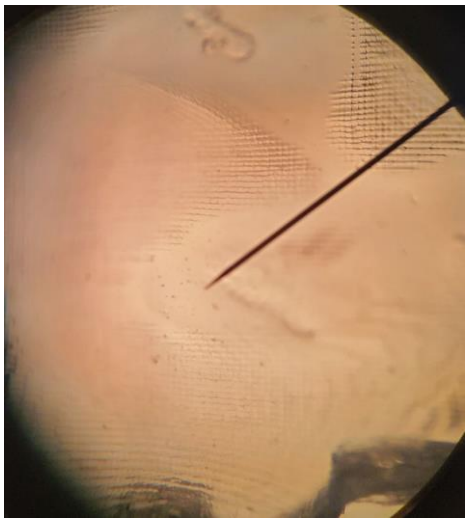


Figure 1- *streptococcus mutans* purity control.

BHI (Brain Heart Infusion) broth was prepared by the manufacturer's instructions.

Eighteen sterile test tubes were filled with 15mL of pure BHI broth, and 0.15mL bacteria ($1x10^{-2}$ dilution). The volume of media was quantified by the size of the cell culture dish (60x15, Thermo-Fisher Scientific), which could contain a maximum of 15mL of fluid. Once dilutions were done, they were incubated at 37°C for 24 hours. Prior to inoculation of the sample, BHI alone, bacteria alone, and BHI and bacteria were streaked on blood agar for a secondary purity control. The sterile aligners samples (N=18) were placed in individual cell culture dishes with 15 mL of BHI and bacteria from test tubes. Test tubes were gently agitated prior to pouring liquid mixture in the cell culture dishes. Dishes were then incubated at 37°C for 48 hours to maximize biofilm formation. Biofilm was observed under 4x magnification with a laboratory microscope (*Figure 2*).

Figure 2

A 

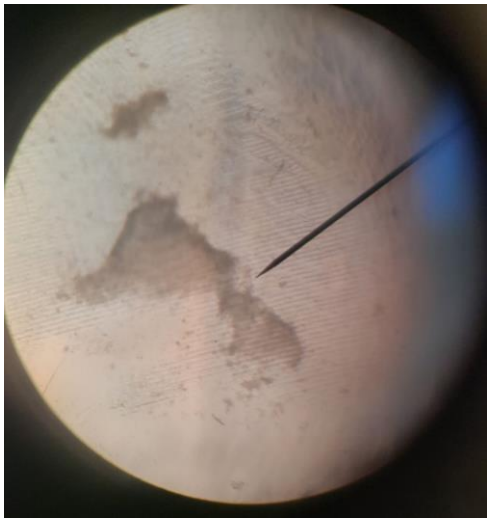
B 

Figure 2a: Biofilm 24 hours under 4x magn.

Figure 2b: Biofilm 48 hours under 4x magn.

After confirming biofilm formation, the samples were cleaned in each respective method explained below, and one microliter sterile loops were used to swab the cusp tips and attachment surface area, ensuring the loop met the entire surface area of previously mentioned. Loop was gently held at 45 degrees to the blood agar plate, moving it back and forth evenly across the entire diameter of the agar plate. Inoculation loops were held at the handle for equal pressure distribution.

Controls: (N=6) Controls were each rinsed under distilled water for 10 seconds. A sterile one microliter loop was used to swab the aligner segment in the orthodontic resin attachment, and cusp tips, and was streaked on a 5% sheep blood agar plate.

Foam: (WhiteFoam, Ingewood, CA) molar segments (N=6) tested using a 10 second rinse under distilled water. As per manufacturer's direction, foam was applied on the intaglio and allowed to sit for 5 minutes prior to swabbing and streaking on a blood agar plate.

Effervescent Tablets: (Retainer Brite, Rochester, MN) molar segments (N=6) tested using a 10 second rinse under distilled water. As per DFU, one tablet was suspended in warm water, aligner

segments were dropped into the suspension for a total of 15 minutes prior to a secondary 10 second rinse under distilled water. Segments were swabbed and streaked on a blood agar plate.

Dish Soap (P&G, West Virginia) molar segments (N=6) were rinsed under distilled water for 10 seconds. A new toothbrush was dipped in Dawn dish soap and aligner pieces were scrubbed for 10 seconds, followed by an additional 10 second rinse under distilled water. Segments were swabbed and streaked on a blood agar plate.

Vinegar molar segments (N=6) were rinsed for 10 seconds under distilled water, and dropped in a suspension of half-warm water, half-white vinegar for 15 minutes prior to an additional 10 second rinse under distilled water. Segments were then swabbed and streaked on a blood agar plate.

Mouthwash (Listerine, J&J, New Jersey) molar segments (N=6) were rinsed for 10 seconds under distilled water and placed in a Listerine solution for 15 minutes prior to a secondary 10 second rinse under distilled water. Segments were swabbed and streaked on a blood agar plate.

After cleansing, each aligner segment was placed in a secondary marked cell culture dish filled with 15mL pure BHI broth. All cell culture dishes were incubated at 37°C for 48 hours, with optical density analysis at 24 hours and 48 hours.

All blood agar plates were marked for identification, placed in an incubation bag with a CO2 generating gas pack, sealed, and incubated for 24 hours at 37degC.

The ten second rinse was a chosen in this experiment, as it was previously demonstrated that after 10 second of rinse with distilled water, a drastic reduction in the quantity of bacteria was observed and it was therefore concluded that a 10 second rinse may be the bottom-line recommendation for cleansing with aligner patients (Porto, et al 2022).

Blood agar plates were analyzed at 24 hours and 48 hours. Colonies were visible and able to be counted at 24 hours. They were enumerated manually and photographed at 24 hours and 48 hours. However, 48-hour microbial counts were not able to be enumerated, as the colony changed in appearance, indicating that S. mutans logarithmic growth would not truly represent the quantity of bacteria present. A 100mL sample was taken from each cell culture dish 24 hours post-clean and 48 hours post-clean, which contained 15mL of broth and the cleaned aligner segment. The samples were observed at each time point in a spectrophotometer (Biotek Synergy 2) at 600nm wavelength.

RESULTS

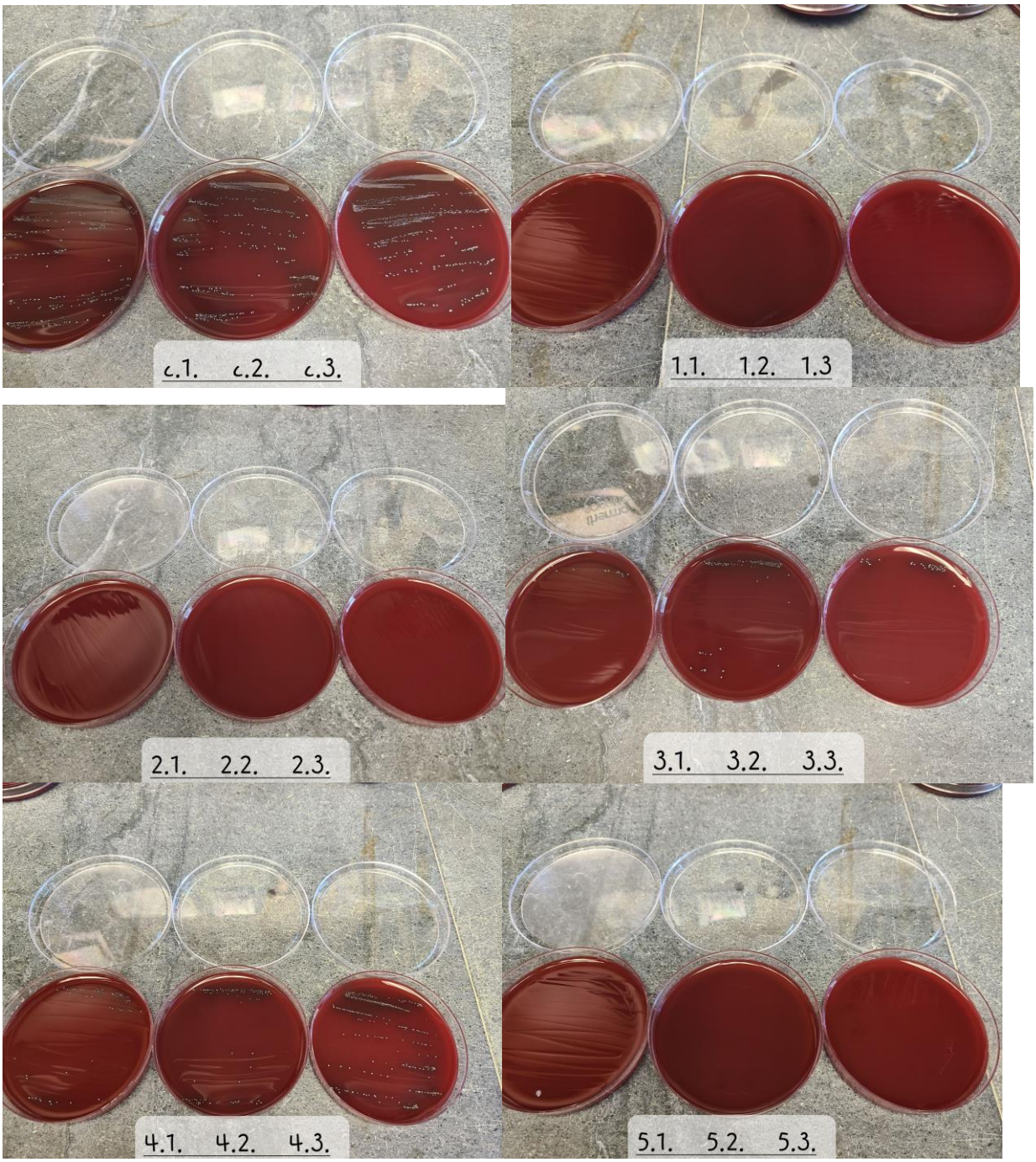
Charts and Photographs

Table I: Mean and SD of bacterial plate counts and OD readings 24h, 48h

	Bacterial Counts		**Optical Density Read**	
	mean	**SD**	**24h**	**48h**
Control	>300	0	0.906	1.157
Foam	0	0	0.142	0.139
Tablet	0	0	0.156	0.15
Dish Soap	117.5	113.52	0.821	1.344
Vinegar	249.17	126.42	0.822	1.81
Mouthwash	0	0	0.156	0.251
			0.129 (BHI media)	0.136 (BHI media)

Table I: *shows that foam, tablet, and mouthwash were successful in removal of S. mutans and dish soap with toothbrush, and vinegar cleansing were not. Density read suggests similar results as the bacterial counts, with additional information on longevity of product success for foam, tablet, and mouthwash.*

Photographs of Plates



Agar plates and bacterial counts. *C: Control 1: Foam 2: Tablets 3: Dish soap 4: Vinegar 5: Listerine*

Optical Density Analysis Charts

24 Hr

	BHI Only	Controls	Foam	Tablets	Dish Soap	Vinegar	Mouthwash
	6	7	8	9	10	11	12

A	0.129	0.93	0.149	0.149	0.467	1.078	0.156
B	0.037	0.967	0.142	0.159	0.807	0.698	0.154
C	0.077	0.822	0.136	0.159	1.19	0.691	0.157
Averages:	**.129**	**0.906**	**0.142**	**0.156**	**0.821**	**0.822**	**0.156**

48 Hr

	BHI Only	**Controls**	**Foam**	**Tablets**	**Dish Soap**	**Vinegar**	**Mouthwash**
	6	7	8	9	10	11	12
F	0.047	0.996	0.14	0.15	2.335	1.708	0.443
G	0.038	0.693	0.141	0.145	0.58	1.403	0.15
H	0.136	1.783	0.138	0.154	1.117	2.318	0.16
Averages:	**.136**	**1.157**	**0.139**	**0.15**	**1.344**	**1.81**	**0.251**

Discussion Tables

Table II: Cost of Products and Ingredients

White Foam	**Retainer Brite**	**Dawn Dish Soap**	**White Vinegar**	**Listerine**
-$19.99 /1/50mL	19.99/ 120tab	$2.99 19.4floz	$1.98 64floz	$5.69 1L
Ingredients: water, hydrogen peroxide, glycerine, PVP, PEG, Sodium lauryl sulfate, sucralose, sodium citrate, sodium benzoate, etidronic acid, metha arvensis	Ingredients: Sodium bicarbonate, citric acid, sodium carbonate, sorbitol, TAED PEG180/PEG8, Sodium Lauryl Sulfoacetate, Magnesium Stereate, FDC 1&2	Ingredients: Water, sodium lauryl sulfate, c10-16 alkyldimethylamine oxide, PPG-26, sodium chloride, sodium hydroxide, PEI- 14 PEG- 24, fragrances, colorant Blue1, c9-11 pareth-8, chloroxylenol, glycerin, propylene glycol, terpineol	Ingredients: Distilled white vinegar, diluted with water to 5% acidity	Ingredients: Water , Alcohol (21.6%), Sorbitol Solution Flavoring, Poloxamer 407, Benzoic Acid, Sodium Saccharin, Sodium Benzoate, D&C Yellow No. 10 Fd&C Green No. 3

Table II*: Ingredients of products used in this study.*