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ADHESION OF CARIOGENIC BACTERIA *Streptococcus mutans* TO DIFFERENT THERMOPLASTICS ALIGNERS MATERIALS – AN IN VITRO STUDY

by

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Submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry

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DEDICATION

I dedicate this work to my two wonderful kids, Arthur and Theo for giving me love unconditionally, make me the best mom for them, I love you so much. You boys are the most important part of my life. I hope all my dedication to my studies, all my strength to support all the difficulties that life imply to us serve you as a guide for your life. Thank you for supporting me through this time. Together we are one and a beautiful small family.

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I would like to thank all my classmates for all support and for being that amazing class that support each other no matter what. Thank you for being my home away from home.

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ADHESION OF CARIOGENIC BACTERIA *Streptococcus mutants* TO DIFFERENT THERMOPLASTICS ALIGNERS MATERIALS – AN IN VITRO STUDY

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BETINA GREHS, DDS

ABSTRACT

Background and Significance: The clear thermoplastic aligner market is the fastest growing segment in orthodontics. There are a variety of materials that can be used to make these aligners. It would be good to know if some are more antibacterial than others.

Purpose: To evaluate differences in the adherence of Strep Mutans to 10 different clear thermoplastics.

Methods: A small section of the molar area from each aligner material was incubated for 24 hours in the presence of Strep Mutans. At the conclusion of the incubation, a sample was taken from each aligner and plated on blood agar. Bacterial colonies harvested from the agar and diluted by 10^9 to allow counting using a calibrated microscope. Similarly, the 10 samples were each rinsed with water for 20 seconds and the colonies counted.

Results: All aligners had billions of bacteria adhering to the plastic after 24 hours. There were no clinically significant differences among the materials. Rinsing for 20 seconds with water dramatically reduced the number of bacteria growing on all aligner materials.

Conclusion: Currently available aligner materials have no antibacterial activity. Rinsing frequently with water is recommended to reduce the risk of dental disease during treatment.

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INTRODUCTION

The popularity of orthodontic treatment for both adults and children has increased in recent years. These treatments usually involves esthetic and metal-free orthodontic devices. (Jeremiah 2011). The introduction of clear aligners has revolutionized orthodontic treatment. There has been an increase in the number of Adults seeking this kind of treatment and more recently teenagers and kids. The clear aligner therapy is made of a series of a thermoformed plastic trays, that are designed to sequentially move teeth in a desired direction. The planning is made by modeling using different softwares. The recommendation is to wear aligners 20-22 hours per day, and remove only for food intake or to brush teeth (Abbate 2015). Because clear aligners are removable appliances they are not considered to interfere with patient's oral hygiene (Miethke 2007); This is an advantage of clear aligners over fixed orthodontic devices, they are easier to clean and facilitate tooth brushing (Levrini 2013 and 2015).

The microbial biofilms are present in the oral environment and are etiologically associated with oral diseases such as caries, gingivitis and periodontitis. The bacteria's and other microorganisms are structured in biofilms that can adhere to solid surfaces such as plastics and tooth enamel. (Costerton 1995 and Dunne 2002). The biofilm is a thin pellicle that covers the enamel surface and indirectly facilitates the adhesion of the bacteria to the tooth surface. (Marsh 1995) The bacteria is imbibed in the biofilm, they become more resistance to any disinfectants products that a non-biofilm bacteria. (Srivastra 2016).

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The presence of orthodontic appliances on an oral cavity changes the condition that was often kept in balance through the multiple roles of saliva. This can increase bacterial populations, pH and salivary flow leading to caries. The clear aligners should be clean before being placed in the mouth to prevent bacterial adhesion, growth which can lead to infections, halitosis and caries disease. The surface of the clear aligners can change depend on their composition. Some Physiochemical factors such as surface, hydrophobicity, and porosity of each plastic material could affect bacterial adherence properties in different ways increasing or decreasing bacterial adhesion. (Papaioannou, W. 2007, Quiryen, M. 1995) In other words a different surface can provide ideal space for more accumulation of biofilm.

Orthodontic devices and not only tooth surfaces, can represent an ideal substrate for biofilm formation. Low et al (2011) reported that the flat surfaces have less biofilm formation and accumulation then the tips and the attachments dimples. The bio-adhesion and plaque formation are dependent of the surface morphology, chemistry and surface electric charge. This could lead to caries and periodontal disease. Not only is the design of the aligner important but also the material structure, which can affect bacterial adhesion, both together had an impact on the oral microenvironment.

Levrini and et al (2016) quantified the amount of bacteria accumulation on the surface of the clear aligners using different cleaning methods. Twenty patients that were under Orthodontic treatment with Invisalign, were evaluated during 6 weeks. The patients were instructed to use different cleaning methods such as: water, brushing with tooth paste and brushing with tooth paste and use of sodium carbonate and sulphate tablet. Bioluminometers was the microbiological choice of analysis of the aligners surface. The

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mechanical removal of the biofilm proved to be effective with brushing and tooth paste, but increased adding the use of the sodium carbonate and sulfate tablet.

The bacteria adhesion and biofilm formation in four different aligners materials were tested using salivary bacteria. The aligners samples were incubated for 2 hours and the control groups were Enamel and metal brackets. The differences between the tested aligners materials are comparable to enamel and metal orthodontic brackets and can be therefore considered for clinical use. No significant differences were found in initial microbial attachment and biofilm formation compared with enamel and metallic brackets.

The clear aligners are removable appliances and it is important that before use in the mouth they should be cleaned. Is recommended to clean the clear aligners to avoid the bacteria accumulation on the surfaces, and avoiding the risks of bacteria accumulation on teeth surfaces and periodontium. The information given to patients is often incomplete and unclear related to how to clean the removable aligners.(Levrini 2016). In the literature very little data is available concerning the biofilm formation, accumulation on the clear aligners surfaces and how patients can be educated to clean their appliances.

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REVIEW OF THE LITERATURE

BIOFILM FORMATION

The composition of bacteria plaque that forms on teeth is certainly stable despite some environmental changes (Marsh 1994). The bacteria is able to adhere to solid surfaces and form a biofilm coat on any surfaces. These bacterial biofilms are present in wet surfaces in nature and can cause environmental problems. (Corsteton 1999).

The mouth environment can be similar to other tract sections of the digestive system and has a characteristic composition in having a resident microflora that develops naturally. The tooth plaque microflora varies in composition at distinct anatomical sites on the tooth for example, in fissures, on approximal surfaces, and in the gingival crevice and changings in the local environmental conditions, can affect the composition of the microflora of mucosal surfaces of the dental plaque.

The early colonizers of the tooth surface include members of the genera *Streptococcus spp., Actinomyces spp, Haemophilus spp., Neisseria spp,* and *Veillonella spp.* (Nyvad and Kilian, 1987). These bacteria adhere to the acquired enamel pellicle by specific and non-specific molecular interactions between adhesins on the cell and receptors on the surface (Gibbons, 1989; Busscher et al., 1992). Once established, the microflora at a site remains relatively stable over time despite regular minor perturbations

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to the oral environment (Marsh, 1989). This stability (termed "microbial homeostasis") results from a dynamic balance of microbial interactions (Sanders and Sanders, 1984).

PLAQUE MICROFLORA, DISEASE AND ORTHODONTICS

The Plaque is formed preferentially at a retentive sites or solid surfaces, unless removed by oral hygiene. As plaque density increases, saliva is less able to penetrate the plaque and protect enamel. Microbial homeostasis can break down, and major shifts in the composition of the microflora can occur. (Marsh 1994) The microcolonies consist of bacterial biofilms accumulate on a surface, and that within these microcolonies the bacteria have developed into organized communities with functional heterogeneity.

A bacterial biofilm is a structured community of bacterial cells enclosed in a polymeric matrix self-produced and adherent to a living surface or not living surface. The Biofilms constitute a protected mode of bacteria growth that allows survival in a hostile environment. (Corsteton1999)

The oral cavity is a rich ecosystem with plenty of microorganisms. Bacteria plaque is the responsible for the stablish and progression diseases as periodontal and caries.

Antagonism is also a major mechanism in maintaining microbial homeostasis in plaque. However, a broke in the microbiological balance can occur, creating conditions for those bacteria’s grow and turns the environment susceptive to diseases. (Marsh 2003) Such diets lead to a rise in the proportions of mutans streptococci and lactobacilli, with a concomitant fall in levels of other streptococci, especially members of the *Streptococcus*

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oralis group, which include *S. sanguis, S. oralis,* and *S. mitis* (Stoppelaar 1970). The metabolism of plaque also changes from a heterofermentative pattern to one in which sugars are converted primarily to lactic acid.

The oral cavity microflora in Orthodontics patients has a strong association with *Streptococcus Mutans* and *lactobacilli* colonization, between another bacterial species that can may cause the development of diseases such as caries, decalcifications, and development of periodontal inflammation. (Pandis 2009)

Observations from clinical and laboratory studies have enabled potential factors to be recognized that may disrupt microbial homeostasis in plaque. (Marsh 1994). The orthodontics material such as fixed (Brackets) or removable (aligners) can affect and contribute to a difference in the balance of the Oral ecosystem. The presence of brackets, attachments, plastic aligner material in intimate contact with the tooth enamel can be related to an increase in gingival inflammation and increases risk of enamel decalcifications. (Pandis 2009) The decalcification of the tooth enamel surface can lead to appearance of white spots and caries lesions (Goerlick 1982, Ogaard 1988).

Orthodontic treatment tends to increase the incidence of developing withe spots lesions because of the plaque formation and accumulation around the brackets and the difficulty of patient to maintain their Oral Hygiene. After bonding orthodontic artifacts, such as brackets or other appliances the count of bacterias increase in a higher number. (Chapman et al 2010).

Pandis and et al 2009 investigate the effects of bracket type (conventional and self-ligation) on the level of *Streptococcus mutans* and total bacterial counts in patient Orthodontically treated. The patient (n=32) were select with fixed appliances in both

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arches. Two saliva samples were collected before the initiation of the treatment and after 2-3 moths of the appliance in mouth. No difference was found in the levels of S. Mutans between conventional and self-ligation brackets in the total bacteria count.

The clear aligners seeking for orthodontic treatment increase with the pass of the year and because they are removable appliances a question that arise is if they could benefit patients related to their Oral health, including facilitate Oral hygiene avoiding the formation of white spots lesion and periodontal inflammation.

New technologies with the digital dentistry is giving the opportunity for patients and orthodontists for new treatment modalities. In 1999 a new Orthodontic appliance made of a polymer was introduced by Align (Invisalign®, Align technology, Santa Clara, California). Using a CAD CAM technology, the plastic removable appliance was able to move teeth according a computer design treatment plan. (Levrini et al 2014 and Miethke 2007).

Albhasi et al 2020, investigate in a randomized controlled trial the relationship between clear aligner (CA) therapy and the development of white spot lesion comparing with fixed Orthodontic appliance (FA) treatment. The participants (N=49) were randomly assigned to the CA group and FA group. Quantitative light induced fluorescence (QLF) images were taken before treatment and after 3 months treatment to measure the loss of fluorescence. The mean amount of fluorescence loss was 0.4% for the CA group and 1.2% for the FA group. The study concluded the Orthodontic treatment with CA and FA can cause enamel demineralization. The CA group develop a larger but shallower white spot, while the FA group develop new lesions but smaller in area. Regardless to

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plaque accumulation the authors found a greater accumulation on the FA group compared with CA group.

Comparing the incidence of white spots lesion (WSLs) among patients treated with aligners and traditional braces Buschang et al (2019) evaluate photographs before and after of 244 patients with clear aligners and 206 with traditional fixed appliance regardless to the changes in Oral hygiene (OH), fluorosis, and WSLs. The authors found that 1.2% of the aligner's patient develop WLSs compared with 26% of the fixed appliances treated patients. While aligners patient developed three new WLSs, traditional fixed treated patients develop 174 WSLs. The incidence of the white spots lesion was greater for maxillary than mandibular and greater in canines than incisors. The authors concluded that aligners have a less risk of developing WSLs than patient undergoing to fixed traditional braces that they attribute to the short treatment time and better pretreatment oral Hygiene.

The prevention of flushing effect of the saliva cause by the use of thermoformed plastic aligners for orthodontic retention was tested by Turkoz et al (2012). Twenty-four patients used Essix aligners for entire for both jaws and the plaque samples were collected after debonding (T0), day 15 (T1), day 30 (T2) and day 60 (T3) of retention. The numbers of Lactobacillus and S. *Mutans* were counted. The authors found the numbers of lactobacillus and *S Mutans* colonies at T3 were higher then T0, T1 and T2 as results, concluding the retention with thermoplastic aligners can proportionate a environment for a grow of *S. Mutans* Lactobacillus on dental surfaces in a high level.

The ability to use clear aligners seems to be better for the patient regards to compliance of oral hygiene procedures. It is very important a correct oral hygiene of to

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impede the bacteria adhesion to tooth surface, avoiding the potential risk for diseases, however the correct cleaning and disinfect plastic removable aligners are necessary as well. There is no Scientific evidence in literature showing any antimicrobial properties of the available vaccuform plastics for clear aligners treatment and a lack of information how patients could clean their aligners to remove the biofilm on the plastic surface.

Levrini and et al (2015) evaluate the most effective method of cleaning removable orthodontic aligners. During six weeks the patients (N=12) were instructed to clean their aligners in e stages: just running in water (control); effervescent tables containing sodium carbonate and sulfate crystals and brushing with toothbrush and brushing with toothbrush and toothpaste. The authors found the brushing combined with use of sodium carbonate and sulfate crystals showed the best results and the brushing only was the least one.

Levrini and et al (2016) quantified the amount of bacteria accumulation on the surface of the clear aligners using different cleaning methods. Twenty patients that were under Orthodontic treatment with Invisalign, were evaluated during 6 weeks. The patients were instructed to use different cleaning methods such as: water, brushing with tooth paste and brushing with tooth paste and use of sodium carbonate and sulphate tablet. Bioluminometers was the microbiological choice of analysis of the aligners surface. The mechanical removal of the biofilm proved to be effective with brushing and tooth paste, but increased adding the use of the sodium carbonate and sulfate tablet.

Lombardo et al (2017) tested nine types of cleaning methods to clean clear aligner (F22) during a period of 14 days in five patients. Patient were instructed to clean the aligners using the instructed strategy: rinsing with water, immersion in water in a sonic

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bath, immersion in water in a ultrasonic bath, immersion in water and anionic detergent in a sonic bath, immersion in water and anionic detergent in a ultrasonic bath, immersion in water and cationic detergent, immersion in water and cationic detergent in a sonic bath and Immersion with water and cationic detergent in a ultrasonic bath, for 5 min twice a day and the aligners were observed with SEM. The authors concluded after evaluate the 9 ways to clean the clear aligner (F22) only the strategy involving 5 min of ultrasound at 42 KHz combined with 0.3% solution of the germicidal cationic detergent benzalkonium chloride is the most effective one to remove the bacterial biofilm.

BIOMATERIAL USED FOR CLEAR ALIGNERS

The thermoplastic aligner system was first introduced by Align Technology (San Jose, CA) during the late 1990s. It was the first aligner system, which allowed for a tooth movements (Kim 2007). The first aligners were made of a single-layer, rigid polyurethane obtained from methylene diphenyl diisocyanato and 1,6-hexanediol (Kim 2007). In 2012, SmartTrack (Align Technology) introduced a thermoplastic polyurethane (TPU) and claimed that it could convey lighter and more uniform forces, and has greater elasticity, making orthodontic tooth movement more predictable. Other aligner manufacturers currently use poly(ethylene terephthalate glycol-modified) (PETG). Nevertheless, polypropylene, polycarbonate, TPU, ethylene vinyl acetate, and many other materials are also used (Dupaix 2005).

Clear aligners are manufactured in 0.50 to 1.5 mm thickness, which also affects their mechanical properties and performance (Guarneri 2013). Since the amount

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of orthodontic force exerted by a clear thermoplastic appliance depends on various properties of the material, a thorough study of different properties of commercially available thermoplastic materials is imperative (Baldwin 2008). The performance of an orthodontic aligner thermoplastic mainly depends on its transparency and mechanical properties, such as strength, modulus and relaxation behavior. Although many studies have reported mechanical studies of aligner materials (Fang 2013), they mainly studied the mechanical properties and less focus was put on the effects of chemical structure on the physical properties. It is important to note that the family of the polymer, such as TPU, PETG, poly(ethylene terephthalate) (PET) or polycarbonate (PC), alone does not tell us much about its exact mechanical properties. Each of them can have very a large range of mechanical properties based on their chemical composition.

For examples, with different hard segments and soft segments, or the relative ratio of them, TPU can have numerous tailored mechanical properties (e.g. Young's modulus from 10 MPa to 10000 MPa) (Elastollan 2012). Based on this reason, although the general composition of some of the aligner materials can be easily found in manufacturer's web site, it is still important to look deeper into the relationship between the chemical structure and physical properties. Fourier transform infrared spectroscopy (FTIR) is used to preliminarily characterize our polymer samples. Attenuated total reflectance-Fourier transform infrared spectrometer provides extremely fast and convenient measurements. With the help of TGA and dissolution test in organic solvent, lamellated composite structure can be found. The chemical composition of them was then further characterized H NMR. Although identifying special additives from the polymers would be very difficult, H NMR is capable of identifying and determining the ratio of

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common components in different types of polymers. After gaining the information about the chemical composition of the materials, differential scanning calorimetry was used to examine the thermal properties, like phase transition and glass transition, of the samples. These properties are closely related to the structure and transparency of these clear thermoplastics. Finally, and most importantly, the flexural moduli of them were measured by dynamic mechanical analysis. Flexural modulus of the thermoplastic directly reflects the performance of the capability of the aligners in delivery orthodontic force. The DSC and DMA results will then be examined and discussed based on the structural information obtained from the chemical analyses.

Kowk and et al (2021) in a in-vitro experimental study, assessed the physical and chemical properties of five different thermoplastic transparent aligner materials as follows: SmartTrack (ST), Zendura, Essix Ace (Ace), Essix C+ (C+), and Tru-Tain DX30-25 (DX30). Dissolution property, thermogravimetric analysis (TGA), nuclear magnetic resonance (NMR) and attenuated total reflectance-Fourier transform Infrared (ATR-FTIR) spectroscopy were measured for chemical evaluation. Differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA) were measured for physical evaluation. We found that C+ was not soluble in any of the tested solvents. Ace and DX30 were soluble in chloroform. ST showed a two-stage degradation according to the TGA results. These results suggest that the core of ST, Ace, and DX30 are PETG or PCT type thermoplastics, while the skin of ST and Zendura are TPU. From the DSC studies, only C+ shows more crystalline characteristic, which is consistent with its lower transparency. The flexural moduli measured by DMA of all 5 aligners are between 0.9 and 1.7 GPa, with Zendura being the stiffest.

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Suter and et al (2020) evaluate the surface roughness and energy of thermoplastic materials used to make orthodontic aligners. Four commercially available thermoplastic materials were selected (CA-medium/CAM, Essix-copopyester/COP, Duran/DUR and Erkodur/ERK). The materials were tested for: reflected light microscopy; optical profilometry for the surface roughness parameters and for the estimation of critical surface tension, total work of adhesion (WA), as well as the work of adhesion due to polar (WP) and dispersion (WD) components employing the Zisman method. Thermoformed disks were prepared and the roughness parameters were calculated before and after thermoformed. Microscopic and profilometric analyses revealed a smooth surface texture in the as-received materials, but a very rough texture after thermoforming, with insignificant differences within each state. Given the differences in surface energy parameters and the lack of roughness differences within the as received or thermoformed groups, it may be concluded that variations in the plaque retaining capacity are anticipated, determined by C, WA and the WP, WD components.

The purpose of this study is based on the fact that the 10 different materials may have different relationships with bacteria, based on their design and physical, mechanical properties. The clear aligner can retain and form a biofilm in a Orthodontic patient, changing the microenvironment and leading to diseases. Knowing a material's susceptibility to bacteria adhesion, allows orthodontists to consider different plastic material available in the market today.

HIPOTHESIS

NULL: There is no differences in bacteria adhesion between the ten different types of plastics.

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PURPOSE: The aims of this study were, firstly, to compare qualitative the colony count of *Streptococcus mutans* to ten commonly used different thermoformed plastic materials used for orthodontic treatment and second to qualitative evaluate the reduction *S. mutans* after rinsing with water for 20 seconds.

MATERIAL AND METHODS

2. Material and Methods

2.1. Samples

2. Material and Methods

This research was performed between August and November of 2021 at Case Western Reserve University (CWRU) Dental School

2.1. Samples

Ten different thermoformed plastics materials were used in this study. Only two of them were preformed by the company and the other eight were prepared from a circle plastic sheet 125mm diameter that were confectioned at the Orthodontic laboratory at Case Western reserve University using pressure molding machine Biostar→ (Scheu-Dental, Germany). This process was performed at the orthodontic laboratory at CWRU

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Dental School. Each sample passed through a thermoformed process: All parameters such as temperature, heating and cooling times are programmed in a reading scanner or entering the corresponding code for each kind of plastic.

The experiments were performed at the research microbiology laboratory at CWRU Dental School.

After all samples were transported to the laboratory in sterile fashion, four pieces from each thermoformed plastic material called (sample) were cut from a molar part in a sterile environment sample (N=10). All the molar pieces were put under UV light for sterilization (Spectrolinker, XL 1000) for 30 minutes upside and 30 minutes downside.



Figure 1 – Molar sample and UV light machine

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Table 1 – Description of the materials

Material	Company	Thickness	Sheet Diameter
1)CWRU Flex H	CWRU	030”	125mm
2)CWRU Flex M	CWRU	030”	125mm
3)CWRU Flex L	CWRU	030”	125mm
4)DX 30-25	Tru tain	030”	125 mm
5)Invisaling Retainers	Align	*	*
6)Zendura™	Bay Materials LLC	030”	125mm
7)Invisalign	Align	*	*
8)Essix C+	Dentsply	040”	125mm
9)Zendura Flex™	Bay Materials LLC	030”	125mm
10)Essix ACE	Dentsply	035”	125mm

*aligner come ready from the company

2.2 Materials

2.2.1 Laboratory equipment including microbiology incubator, refrigerator and Bunsen burner were presented at CWRU dental school microbiology research department and were use following laboratory guidelines, biosafety standards, and protocols by CWRU Dental School. All working surfaces were cleaned and disinfected prior to the experiments per laboratory protocols.

2.2.2 Brain heart Infusion broth (Remel R452472) is a general-purpose liquid medium used for the culture of microorganisms, including aerobic and anaerobic bacteria, yeast, and molds from a variety of clinical and non-clinical specimens. The 37g of BHI were

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suspended in one liter of distilled water, dispensed into a sterile bottle, and put to sterilization in an autoclave for 35 minutes.

2.2.3 Blood Agar plates (Thermo Scientific™ Blood agar – 5% sheep blood)

2.2.4 Disposable inoculating Loops (Fisherbrand®) sterile pre-packaged

2.2.5 Sterile micropipettes tips (Sharp™, Denville)

2.2.6 CO_2 Gas Generating pouch system (BD GasPak EZ™)

2.2.7 McFarland 0.5 (Hardy Diagnostics)

2.2.8 Cell culture plate (Thermo Scientifc™ sterilin™)

2.2.9 Bacteria Streptococcus mutans Culti-loops™ ATCC 25175 (Thermo Fisher Scientific). Streptococcus mutans is a facultatively anaerobic gram-positive cocci. Culti-loops™ was stored at 2-8ºC individually packed in a foil pouch and following manufacture instruction the loop was place in 1mL of Brain heart Infusion broth (BHI) and incubated for 24 hours at 37ºC. After completing 24 hours of incubation 10 µL S. mutans culture were aliquoted for daily use in 10mL BHI at turbidity McFarland 0.5 in sterile glass tubes with cap and incubated 24 hours. For quality control purity culture after incubation for 24 hours was plated on blood agar and placed in CO_2 generation pouch.

Culture characteristics were inspected: small white colonies. (figure 2) Gram stain as gram positive cocci in chains and coagulase negative all these were performed for quality control (QC)

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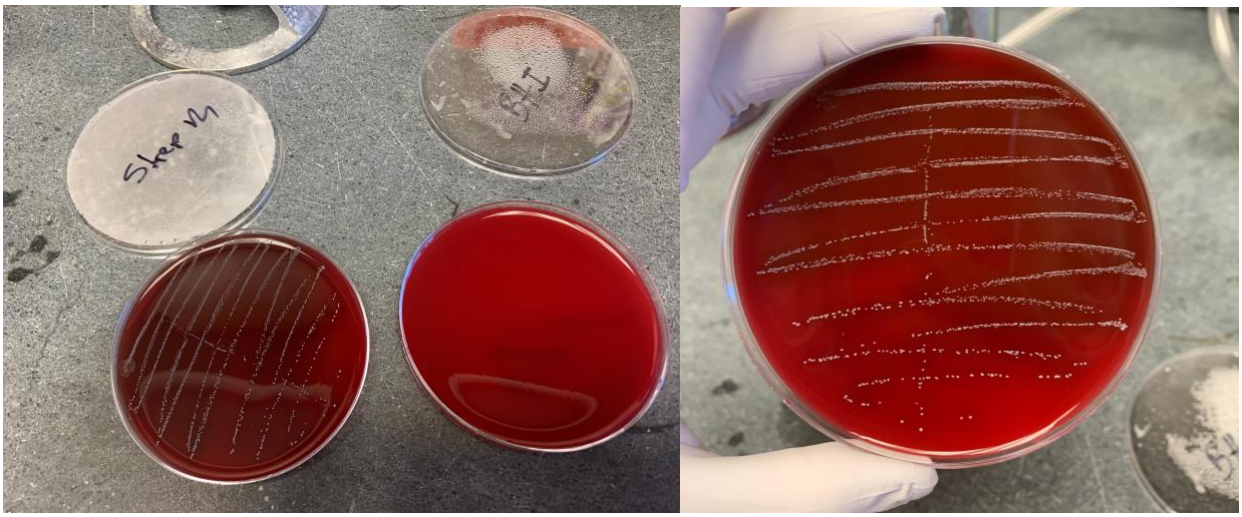


Figure 2 – *Streptococcus mutans* from Tube 1 and BHI control agar plates

2.3 Inoculation

A sterile thermoformed plastic material molar piece (sample) total (N=10). Each sample was placed on a sterile cell culture plate with 15mL of BHI and were inoculated with 10µL of pure *Streptococcus mutans* culture using a micropipette sterile tip. The samples were processed in triplicates for standardization. For quality control three controls were made: First control was for BHI sterility, second control was a thermoformed aligner sample on BHI for sterility of sample, and third control was *Streptococcus mutans* on BHI for purity of the bacteria strain. These controls were run every experiment. Once the bacteria were inoculated into the petri dishes and the controls were made, they were placed in an incubator at 37 degrees Celsius for 24 hours to grow.

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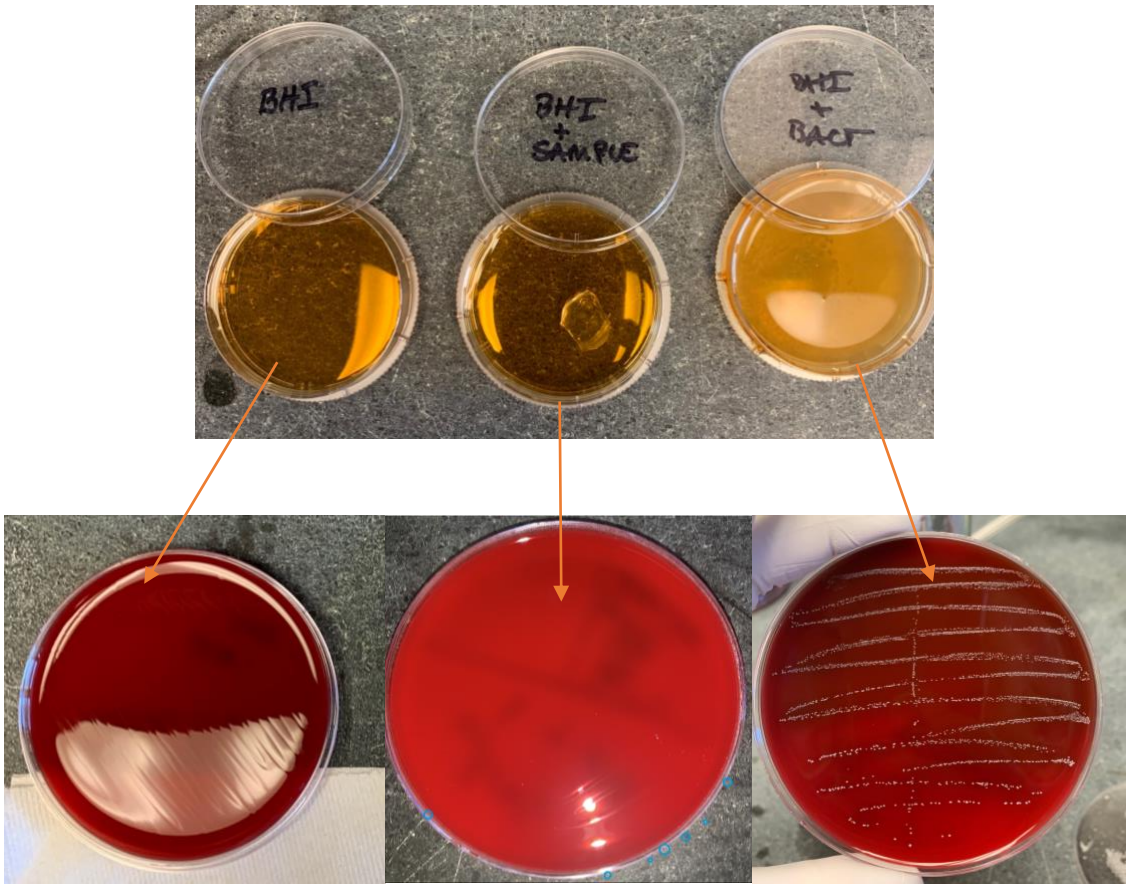


Figure 3 – Controls samples

Completed 24 hours the samples were removed from the incubator and the biofilm formation was checked and pictures were saved.

After 24h controls were inspected and were streaked with 1µL loop in blood agar incubated in CO_2 generating pouch a 37ºC for 24h.

2.4. Quantitative growth on *Streptococcus mutans* in thermoformed plastic material molar pieces

The number of bacteria were reported based on the quantification of colony-forming units (CFU) per mL of sample. For this, several serial dilutions were prepared of

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the sample, plated the diluted suspensions, and count the number of colonies forming units.

The dilutions were streaked each with a new 1µL disposable sterile loop in blood agar for colony count. For transfer each sample dilution we used a new sterile micropipettes tip to avoid contamination.

Using a different sterile tweezer to hold each plastic material a series of dilutions parting from 1µL of biofilm called (biofilm sample). T taking with a 1µL disposable sterile loop from the buccal surface molar piece of thermoformed plastic material. Them serial dilutions in tubes of the biofilm sample suspended on 0.999mL of BHI adjusted to room temperature. Using new sterile micropipette tip for each transfer. See process and diagram below.

1. The first tube contains 1µL of biofilm sample from the molar piece on 0.999 mL of BHI. (D1). This provides an initial dilution of $10^{-3.}$
2. From the first tube 1µL of properly mixed sample/culture is drawn into the micropipette. The sample is then added to the second tube (D2) to make the total volume of 1mL. The dilution is mixed by emptying and filling the pipette several times. Micropipette tip is discarded, and a new pipette is attached to the pipette. D2 now has a total dilution factor as 10^{-6}
3. Then 1µL of mixture is taken from D2 and is emptied into the third tube (D3). The third tube (D3) now has a total dilution factor as 10^{-9}

All three tubes for one sample were streaked each with a new 1µL sterile disposable loop, in one plate on blood agar.

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All the blood agar plates were identified and placed inside CO_2 generating pouch. These were sealed and placed in the incubator at 37^{o}C for 24 hours to allow the cultures to grow. At 24 h the pouches were removed from incubator and the cultures were inspected evaluate colony characteristics, and colony count in CFU. All controls were evaluated at same time.

For quality control 1µL of the control of *S. mutans* serial dilution was conducted. In addition of serial dilution for colony counts three imprints were made for each sample. From the occlusal part of the thermoformed plastic material for each samples on blood agar were made of each sample. See sample picture below.

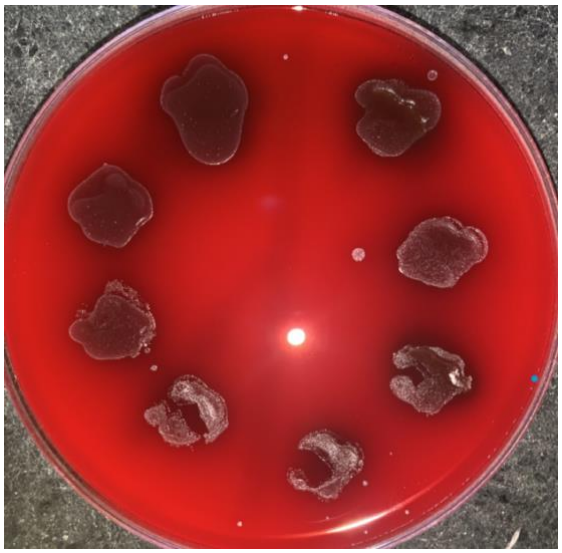


Figure 4 - Blood agar plate imprints

The McFarland Standard 0.5 was used as a reference to adjust the turbidity of the bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing. The standard can be compared visually to a suspension of bacteria in sterile broth. A 0.5 McFarland standard is comparable to a bacterial suspension of 1.5 x 10^{-8} CFU/mL.

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Figure 5- McFarland scale

2.5 Post wash quantitative growth on *Streptococcus mutans* in thermo aligners molar pieces. The inoculation process was repeated for post wash for the samples thermoformed sterile plastic material (N=10) again samples were process by triplicates, with quality controls for BHI sterility, sample sterility, *Streptococcus mutans* purity and additional control for water sterility.

Water cultures: from the laboratory faucet and from commercial osmotic filtrated water were inoculated in BHI and direct streak on 5% blood agar, identified placed in CO2 generating bag and sealed incubated for 24 hours at 37C inspected and pictures were saved. From the BHI cultures with a 10 µL sterile loop were streaked in 5% sheep blood agar plate, identified placed in CO2 generating bag and sealed incubated for 24 hours at 37C inspected and pictures were saved

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After samples and control cultures were inoculated and allow to growth for 24 hours at 37C they were inspected for biofilm. Each sample was removed with sterile tweezers and rinsed for 20 second using osmotic filtrated commercial water (DISTILLATA, spring water). Three imprints from the occlusal surface of the thermoformed plastic material after washing the samples were made on blood agar.

The biofilm from the buccal part of the sample was collected again the same fashion and processed in serial dilution for colony count on CFU.

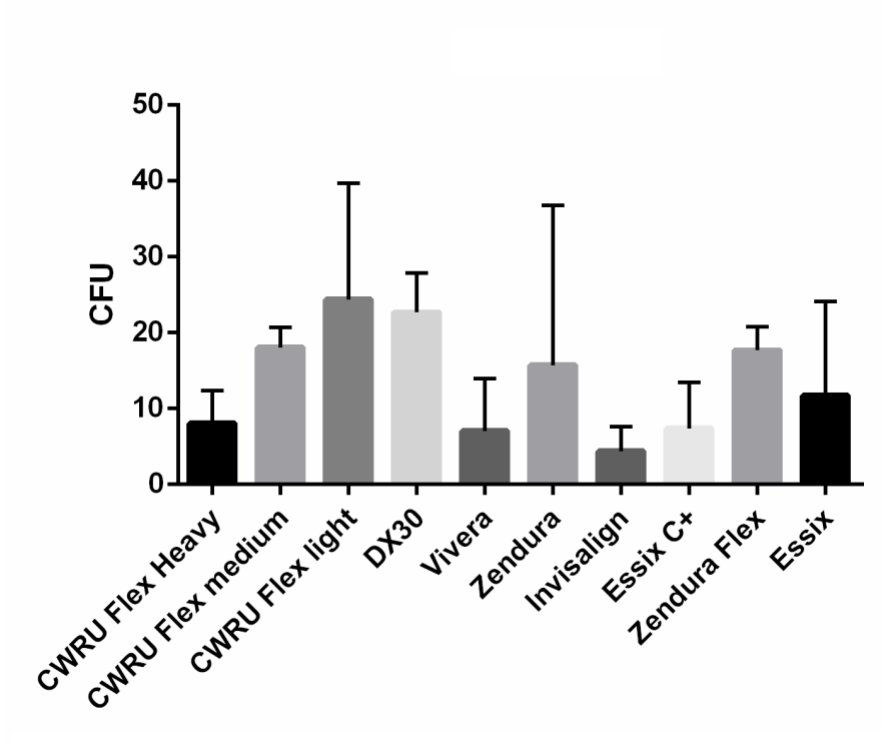
3. Statistical analysis

A descriptive Statistical analysis were performed to describe the basic features of the data in this study. A Excel spreadsheet was used to collection of data and process of average, mean, min, maximum and standard deviation and the program Graphpad Prism was used to produce the graphics.

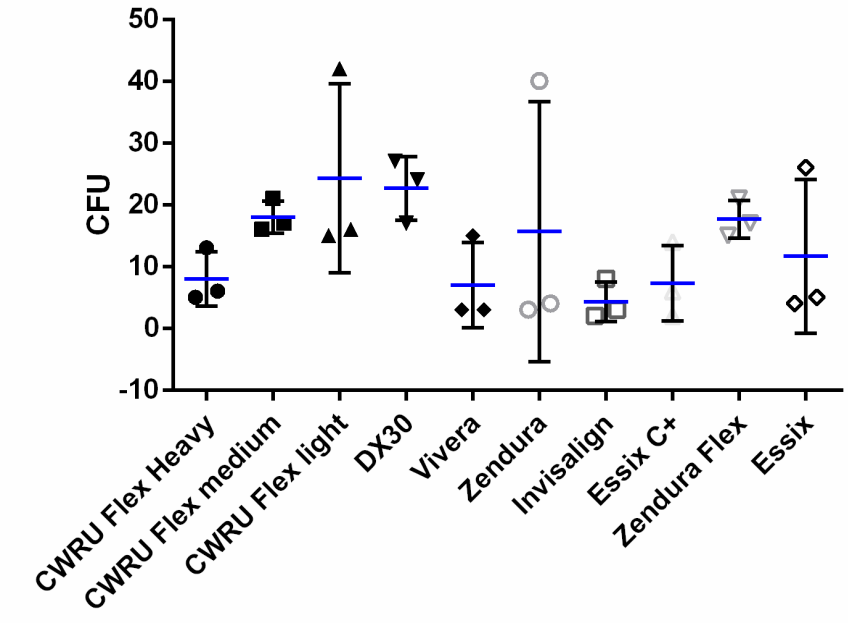
4. Results

Streptococcus mutans grew in appropriate condition on BHI forming biofilm in all (N=10) thermoformed plastic material samples tested in 24 hours. The levels of growth in the imprints and on inoculation were too many to count in all samples in the first two dilutions. On the third dilution Invisalign had the lowest colony count, follow by Invisalign RT, Essix C+, CWRU heavy, Essix ACE, Zendura and Zendura Flex. The worst were CWRU flex light and DX30. See Graph 1.

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Graph 1 – Graphic Adhesion of S. Mutans between the 10 different materials



Graph 2 - Box plot displaying the difference between the 10 types of thermoformed plastic materials

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Table 2. Mean and Standard Deviation for the 10 different type of thermoformed plastic aligners for the dilution 3 (10^9) on CFU (colony-forming unit)

Material	n	mean	SD
CWRU Flex Heavy	3	8,0	4,36
CWRU Flex medium	3	18,0	2,65
CWRU Flex light	3	24,3	15,31
DX30	3	22,7	5,13
Vivera	3	7,0	6,93
Zendura	3	15,7	21,08
Invisalign	3	4,3	3,21
Essix C+	3	7,3	6,11
Zendura Flex	3	17,7	3,06
Essix Ace	3	11,7	12,42

In the post rinse with water the colony counts decreased, initially with water from the faucet all sample including control had a contamination with an environmental bacillus like bacteria (Figure 6).

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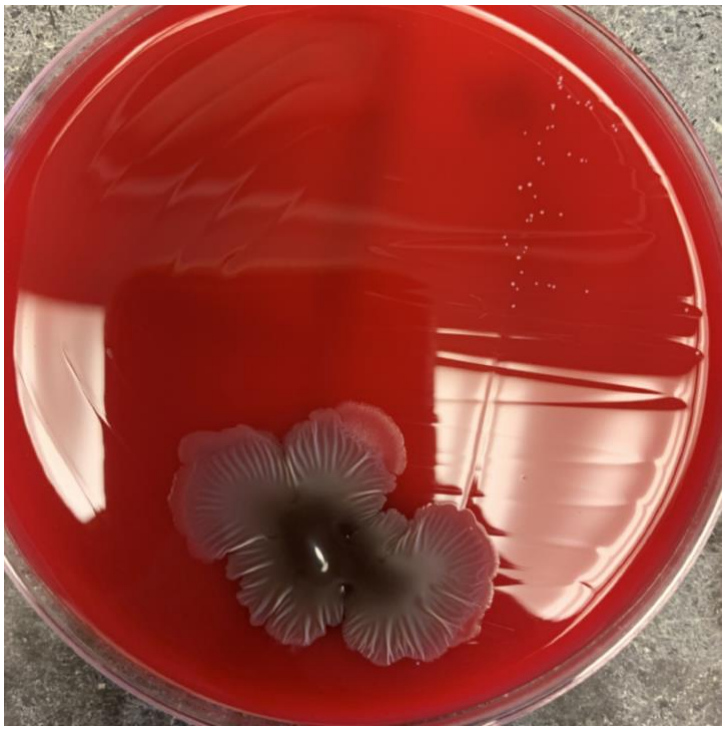
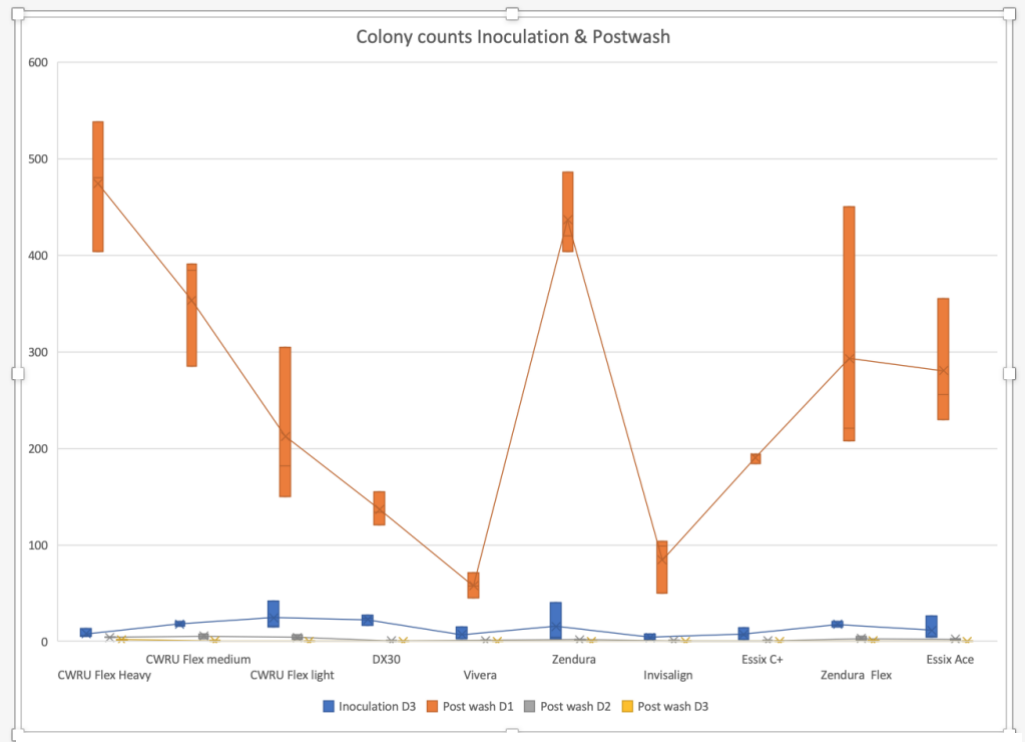


Figure 6 – Contamination for the water faucet after wash

We replaced faucet water for osmotic filtrated commercial water for rinsing the samples for 20 seconds, all colony counts decreased significantly, very low numbers, especially in the second and third dilutions. The three samples CWRU flex heavy, medium and light in the second dilution had $4x10^6$CFU, $5 x10^6$CFU and $2 x10^6$ all other samples had 1 to the 10^6 CFU. In the third dilution most sample went to zero in colony count except for CWRU flex heavy 2 to 10^9, and medium and Zendura flex had a decrease 1 to 10^9 each. See graphic 2.

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Graph2 – Colony count Inoculation and Postwash

Table 3. Mean and Standard Deviation for the 10 different type of thermoformed plastic aligners for the dilution 1 (10^3), D2 (10^6) and D3 (10^9)on CFU (colony-forming unit) after wash with water.

Material	dilution					
	1st /10^3		2nd / 10^6		3rd / 10^9	
	mean	SD	mean	SD	média	dp
CWRU Flex Heavy	474.0	67.20	4.0	0.00	1.7	0.58
CWRU Flex medium	353.3	59.28	5.3	2.52	0.3	0.58
CWRU Flex light	212.3	81.83	4.0	2.65	-	-
DX30	136.7	17.16	0.3	0.58	-	-
Vivera	57.7	13.01	1.0	0.00	-	-
Zendura	436.7	43.47	1.7	0.58	-	-
Invisalign	84.3	29.84	0.7	0.58	-	-
Essix C+	190.3	5.51	0.7	0.58	-	-
Zendura Flex	293.0	136.12	3.0	2.00	0.3	0.58
Essix Ace	280.3	65.96	2.0	1.00	-	-

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All quality control performed well, including purity control in each batch of *Streptococcus mutans* BHI and blood agar sterility plates. Also, after switching to commercial water sterility plate did not growth bacteria. The UV light 30 minutes upside up and 30 minutes up side up was enough to sterilize the plastic thermoformed materials for the experiments.

5. Discussion

Based on the experimental results of the present study, the null hypothesis was rejected for the bacteria adhesion since significant colonies formation were encountered in the surface of the different plastics.

In the present study, ten different thermoformed plastic materials were used for determination of the surface adherence of Streptococcus mutans. The main reasons were determining between of the most commonly used plastic material for aligners and retainers which one had an ability to grow more bacteria in an In vitro ideal condition or to see if any of those material had any antimicrobial properties. All tests were performed using brain heart infusion broth at 37^{o}C for 24 hs.

Comparing the adherence of *Streptococcus mutans* on ten commonly use tested thermoformed plastic materials all grew bacteria in an in vitro ideal condition simulating oral cavity brain heart infusion broth at 37for 24 hours. They testes samples displayed significant difference with the best performance on Invisalign®.

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Rinsing thermoformed plastic material with clean water for 20 seconds significantly decreased the colony count invitro control study, implying that this will decrease the number of bacteria in the surface of the removable appliance in real life.

The difference present in the dilution when comparing each sample against itself (triplicates) and It was only present on the dilutions and not in the imprints, It was presume effect of the location in the molar buccal part where the sample was collected (more bacteria on some deeper molar parts than others), and these difference were less evident after water rinse. The difference disappeared on the second and third dilution after water rinse per say an effect of a dilution.

Limited information is available to recommend patient how to clean their removable thermoformed orthodontic appliances, these results suggest rinsing with clean water will significantly decrease the amount *Streptococcus mutans* on the surface of the thermoformed plastic material. Likely frequent rinses will effectible and safely decrease the number of bacteria growing in the surface of the orthodontic removable appliance without affecting the physicochemical characteristic of the appliance. Timed studies for optimization of rinsing time with clean water.

Patient should we informed of need of clean water use. Use of contaminated water can caused growth of environmental bacterial in the thermoformed plastic material, from invitro observation. It is unclear it this bacterial will growth will appliance are place into oral cavity due to normal defense mechanism of the saliva and oral cavity physiology.

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In real time appliance are wear usually for shorter periods of time that varied in each patient from 2 to 12 hours and the recommended add total time in a day is 20 to 22 hours. Invitro 24 hours growth was enough time for pure *Streptococcus mutans* to form a biofilm on the surface of appliance without competing with other bacteria, candida ssp. Other microorganism and inhibitory condition of saliva, mastication, etc.

6. Conclusion

The results of this study suggest that all the different plastic aligners tested do not have any antimicrobial properties that can avoid biofilm formation during the tested period of 24 hours. The thermoformed plastic that had the best performance regardless S. Mutans adherence was Invisalign aligners and the one who absorbed more was CWRU flex light followed by DX30. After wash with water we noticed a decrease in bacteria count with CWRU flex heavy the one who showed less decrease followed by CWRU flex medium and Zendura Flex.

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STATEMENT OF THESIS

The aims of this study were, firstly, to compare qualitative the colony count of Streptococcus mutans to ten commonly used different thermoformed plastic materials used for orthodontic treatment and second to qualitative evaluate the reduction S. mutans after rinsing with water for 20 seconds.

Hypotheses:

1. There is a significant difference in a adhesion of Streptococcus Mutans in the ten different types of thermoformed plastic aligner materials

2. There is no a significant difference in a adhesion of Streptococcus Mutans in the ten different types of thermoformed plastic aligner materials

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ADHESION OF CARIOGENIC BACTERIA Streptococcus mutants TO DIFFERENT THERMOPLASTICS ALIGNERS MATERIALS – AN IN VITRO STUDY

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ABSTRACT

Background and Significance: The clear thermoplastic aligner market is the fastest growing segment in orthodontics. There are a variety of materials that can be used to make these aligners. It would be good to know if some are more antibacterial than others.

Purpose: To evaluate differences in the adherence of Strep Mutans to 10 different clear thermoplastics.

Methods: A small section of the molar area from each aligner material was incubated for 24 hours in the presence of Strep Mutans. At the conclusion of the incubation, a sample was taken from each aligner and plated on blood agar. Bacterial colonies harvested from the agar and diluted by 10^9 to allow counting using a calibrated microscope. Similarly, the 10 samples were each rinsed with water for 20 seconds and the colonies counted.

Results: All aligners had billions of bacteria adhering to the plastic after 24 hours. There were no clinically significant differences among the materials. Rinsing for 20 seconds with water dramatically reduced the number of bacteria growing on all aligner materials.

Conclusion: Currently available aligner materials have no antibacterial activity. Rinsing frequently with water is recommended to reduce the risk of dental disease during treatment.

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INTRODUCTION

The popularity of orthodontic treatment for both adults and children has increased in recent years. These treatments usually involves esthetic and metal-free orthodontic devices. (Jeremiah 2011). The introduction of clear aligners has revolutionized orthodontic treatment. There has been an increase in the number of Adults seeking this kind of treatment and more recently teenagers and kids. The clear aligner therapy is made of a series of a thermoformed plastic trays, that are designed to sequentially move teeth in a desired direction. The planning is made by modeling using different softwares. The recommendation is to wear aligners 20-22 hours per day, and remove only for food intake or to brush teeth (Abbate 2015). Because clear aligners are removable appliances they are not considered to interfere with patient's oral hygiene (Miethke 2007); This is an advantage of clear aligners over fixed orthodontic devices, they are easier to clean and facilitate tooth brushing (Levrini 2013 and 2015).

The microbial biofilms are present in the oral environment and are etiologically associated with oral diseases such as caries, gingivitis and periodontitis. The bacteria's and other microorganisms are structured in biofilms that can adhere to solid surfaces such as plastics and tooth enamel. (Costerton 1999 and Dunne 2002). The biofilm is a thin pellicle that covers the enamel surface and indirectly facilitates the adhesion of the bacteria to the tooth surface. (Marsh 1995) The bacteria is imbibed in the biofilm, they becomemore resistance to any disinfectants products that a non-biofilm bacteria. (Srivastra 2016).

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The presence of orthodontic appliances on an oral cavity changes the condition that was often kept in balance through the multiple roles of saliva. This can increase bacterial populations, pH and salivary flow leading to caries. The clear aligners should be clean before being placed in the mouth to prevent bacterial adhesion, growth which can lead to infections, halitoses and caries disease. The surface of the clear aligners can change depend on their composition. Some Physiochemical factors such as surface, hydrophobicity, and porosity of each plastic material could affect bacterial adherence properties in different ways increasing or decreasing bacterial adhesion. (Papaioannou, W. 2007, Quiryen, M. 1995) In other words a different surface can provide ideal space for more accumulation of biofilm.

Orthodontic devices and not only tooth surfaces, can represent an ideal substrate for biofilm formation. Low et al (2011) reported that the flat surfaces have less biofilm formation and accumulation then the tips and the attachments dimples. The bio-adhesion and plaque formation are dependent of the surface morphology, chemistry and surface electric charge. This could lead to caries and periodontal disease. Not only is the design of the aligner important but also the material structure, which can affect bacterial adhesion, both together had an impact on the oral microenvironment.

Levrini and et al (2016) quantified the amount of bacteria accumulation on the surface of the clear aligners using different cleaning methods. Twenty patients that were under Orthodontic treatment with Invisalign, were evaluated during 6 weeks. The patients were instructed to use different cleaning methods such as: water, brushing with tooth paste and brushing with tooth paste and use of sodium carbonate and sulphate tablet. Bioluminometers was the microbiological choice of analysis of the aligners surface. The

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mechanical removal of the biofilm proved to be effective with brushing and tooth paste, but increased adding the use of the sodium carbonate and sulfate tablet.

The bacteria adhesion and biofilm formation in four different aligners materials were tested using salivary bacteria. The aligners samples were incubated for 2 hours and the control groups were Enamel and metal brackets. The differences between the tested aligners materials are comparable to enamel and metal orthodontic brackets and can be therefore considered for clinical use. No significant differences were found in initial microbial attachment and biofilm formation compared with enamel and metallic brackets. The aims of this study were, firstly, to compare qualitative the colony count of Streptococcus mutans to ten commonly used different thermoformed plastic materials used for orthodontic treatment and second to qualitative evaluate the reduction S. mutans after rinsing with water for 20 seconds.

MATERIAL AND METHODS

2.1. Samples

Ten different thermoformed plastics materials were used in this study. Only two of them were preformed by the company and the other eight were prepared from a circle plastic sheet 125mm diameter that were confectioned at the Orthodontic laboratory at Case Western reserve University using pressure molding machine Biostar (Scheu-Dental, Germany). This process was performed at the orthodontic laboratory at CWRU Dental School. Each sample passed through a thermoformed process: All parameters such as temperature, heating and cooling times are programmed in a reading scanner or entering the corresponding code for each kind of plastic.

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The experiments were performed at the research microbiology laboratory at CWRU Dental School.

After all samples were transported to the laboratory in sterile fashion, four pieces from each thermoformed plastic material called (sample) were cut from a molar part in a sterile environment sample (N=10). All the molar pieces were put under UV light for sterilization (Spectrolinker, XL 1000) for 30 minutes upside and 30 minutes downside.

Table 1- Material description

Material	Company	Thickness	Sheet Diameter
1)CWRU Flex H	CWRU	030”	125mm
2)CWRU Flex M	CWRU	030”	125mm
3)CWRU Flex L	CWRU	030”	125mm
4)DX 30-25	Tru tain	030”	125 mm
5)Invisaling Retainers	Align	*	*
6)Zendura™	Bay Materials LLC	030”	125mm
7)Invisalign	Align	*	*
8)Essix C+	Dentsply	040”	125mm
9)Zendura Flex™	Bay Materials LLC	030”	125mm
10)Essix ACE	Dentsply	035”	125mm

2.2 Materials

2.2.1 Laboratory equipment including microbiology incubator, refrigerator and Bunsen burner were presented at CWRU dental school microbiology research department and were use following laboratory guidelines, biosafety standards, and protocols by CWRU Dental School. All working surfaces were cleaned and disinfected prior to the experiments per laboratory protocols.

2.2.2 Brain heart Infusion broth (Remel R452472) is a general-purpose liquid medium used for the culture of microorganisms, including aerobic and anaerobic bacteria, yeast,

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and molds from a variety of clinical and non-clinical specimens. The 37g of BHI were suspended in one liter of distilled water, dispensed into a sterile bottle, and put to sterilization in an autoclave for 35 minutes.

2.2.3 Blood Agar plates (Thermo Scientific™ Blood agar – 5% sheep blood)

2.2.4 Disposable inoculating Loops (Fisherbrand®) sterile pre-packaged

2.2.5 Sterile micropipettes tips (Sharp™, Denville)

2.2.6 CO_2 Gas Generating pouch system (BD GasPak EZ™)

2.2.7 McFarland 0.5 (Hardy Diagnostics)

2.2.8 Cell culture plate (Thermo Scientifc™ sterilin™)

2.2.9 Bacteria Streptococcus mutans Culti-loops™ ATCC 25175 (Thermo Fisher Scientific). Streptococcus mutans is a facultatively anaerobic gram-positive cocci. Culti-loops™ was stored at 2-8ºC individually packed in a foil pouch and following manufacture instruction the loop was place in 1mL of Brain heart Infusion broth (BHI) and incubated for 24 hours at 37ºC. After completing 24 hours of incubation 10 µL S. mutans culture were aliquoted for daily use in 10mL BHI at turbidity McFarland 0.5 in sterile glass tubes with cap and incubated 24 hours. For quality control purity culture after incubation for 24 hours was plated on blood agar and placed in CO_2 generation pouch.

Culture characteristics were inspected: small white colonies. (See picture below). Gram stain as gram positive cocci in chains and coagulase negative all these were performed for quality control (QC). 2.3 Inoculation. A sterile thermoformed plastic material molar piece (sample) total (N=10). Each sample was placed on a sterile cell culture plate with 15mL of BHI and were inoculated with 10µL of pure Streptococcus mutans culture using a micropipette sterile tip. The samples were processed in triplicates

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for standardization. For quality control three controls were made: First control was for BHI sterility, second control was a thermoformed aligner sample on BHI for sterility of sample, and third control was Streptococcus mutans on BHI for purity of the bacteria strain. These controls were run every experiment. Once the bacteria were inoculated into the petri dishes and the controls were made, they were placed in an incubator at 37 degrees Celsius for 24 hours to grow.

Completed 24 hours the samples were removed from the incubator and the biofilm formation was checked and pictures were saved. After 24h controls were inspected and were streaked with 1µL loop in blood agar incubated in CO_2 generating pouch a 37^{o}C for 24h.

2.4. Quantitative growth on Streptococcus mutans in thermoformed plastic material molar pieces

The number of bacteria were reported based on the quantification of colony-forming units (CFU) per mL of sample. For this, several serial dilutions were prepared of the sample, plated the diluted suspensions, and count the number of colonies forming units.

The dilutions were streaked each with a new 1µL disposable sterile loop in blood agar for colony count. For transfer each sample dilution we used a new sterile micropipettes tip to avoid contamination.

Using a different sterile tweezer to hold each plastic material a series of dilutions parting from 1µL of biofilm called (biofilm sample). Taking with a 1µL disposable sterile loop from the buccal surface molar piece of thermoformed plastic material. Them

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serial dilutions in tubes of the biofilm sample suspended on 0.999mL of BHI adjusted to room temperature. Using new sterile micropipette tip for each transfer. See process and diagram below.

1. The first tube contains 1µL of biofilm sample from the molar piece on 0.999 mL of BHI. (D1). This provides an initial dilution of $10^{-3.}$
2. From the first tube 1µL of properly mixed sample/culture is drawn into the micropipette. The sample is then added to the second tube (D2) to make the total volume of 1mL. The dilution is mixed by emptying and filling the pipette several times. Micropipette tip is discarded, and a new pipette is attached to the pipette. D2 now has a total dilution factor as 10^{-6}
3. Then 1µL of mixture is taken from D2 and is emptied into the third tube (D3). The third tube (D3) now has a total dilution factor as 10^{-9}

All three tubes for one sample were streaked each with a new 1µL sterile disposable loop, in one plate on blood agar. All the blood agar plates were identified and placed inside CO_2 generating pouch. These were sealed and placed in the incubator at 37^{o}C for 24 hours to allow the cultures to grow. At 24 h the pouches were removed from incubator and the cultures were inspected evaluate colony characteristics, and colony count in CFU. All controls were evaluated at same time. For quality control 1µL of the control of S. mutans serial dilution was conducted. In addition of serial dilution for colony counts three imprints were made for each sample. From the occlusal part of the thermoformed plastic material for each samples on blood agar were made of each sample.

The McFarland Standard 0.5 was used as a reference to adjust the turbidity of the bacterial suspensions so that the number of bacteria will be within a given range to

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standardize microbial testing. The standard can be compared visually to a suspension of bacteria in sterile broth. A 0.5 McFarland standard is comparable to a bacterial suspension of 1.5 x 10^{-8} CFU/mL.

2.5 Post wash quantitative growth on Streptococcus mutans in thermo aligners molar pieces: The inoculation process was repeated for post wash for the samples thermo formed sterile plastic material (N=10) again samples were process by triplicates, with quality controls for BHI sterility, sample sterility, Streptococcus mutans purity and additional control for water sterility. Water cultures: from the laboratory faucet and from commercial osmotic filtrated water were inoculated in BHI and direct streak on 5% blood agar, identified placed in CO2 generating bag and sealed incubated for 24 hours at 37C inspected and pictures were saved. From the BHI cultures with a 10 µL sterile loop were streaked in 5% sheep blood agar plate, identified placed in CO2 generating bag and sealed incubated for 24 hours at 37C inspected and pictures were saved.

After samples and control cultures were inoculated and allow to growth for 24 hours at 37C they were inspected for biofilm. Each sample was removed with sterile tweezers and rinsed for 20 second using osmotic filtrated commercial water (Distillata spring water). Three imprints from the occlusal surface of the thermoformed plastic material after washing the samples were made on blood agar. The biofilm from the buccal part of the sample was collected again the same fashion and processed in serial dilution for colony count on CFU.

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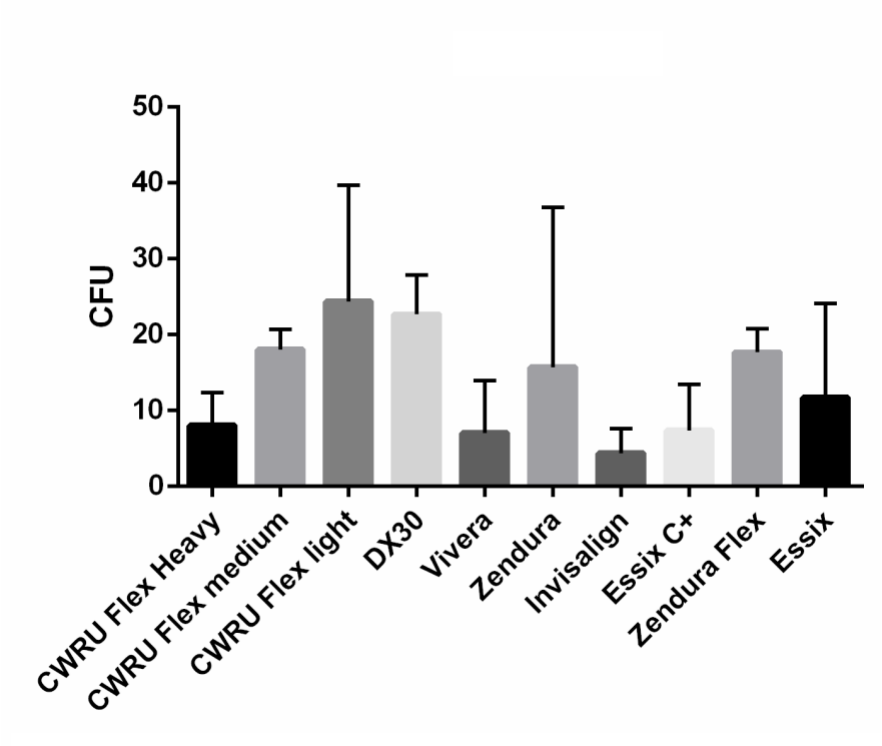
3. Statistical analysis

A descriptive Statistical analysis were performed to describe the basic features of the data in this study. A Excel spreadsheet was used to collection of data and process of average, mean, min, maximum and standard deviation and the program Graphpad Prism was used to produce the graphics.

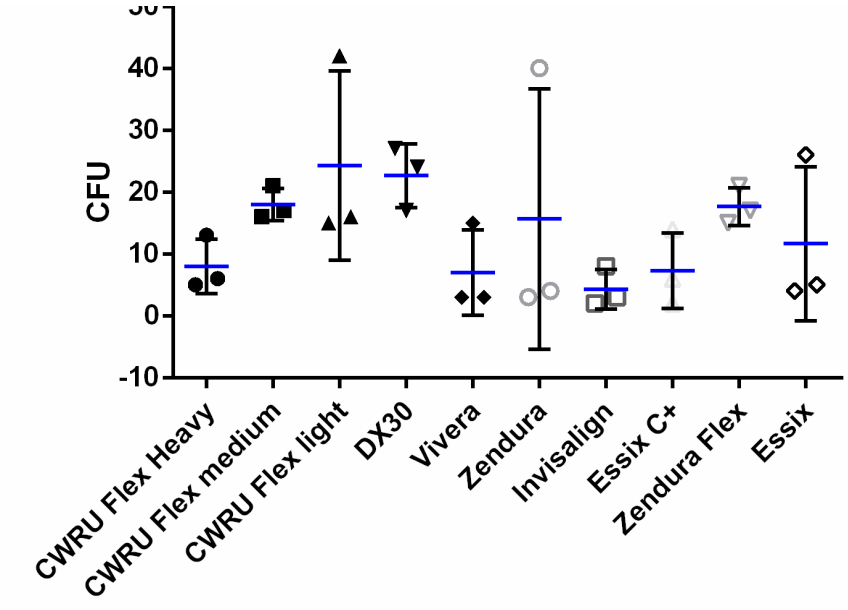
4. Results

Streptococcus mutans grew in appropriate condition on BHI forming biofilm in all (N=10) thermoformed plastic material samples tested in 24 hours. The levels of growth in the imprints and on inoculation were too many to count in all samples in the first two dilutions. On the third dilution Invisalign had the lowest colony count, follow by Invisalign RT, Essix C+, CWRU heavy, Essix ACE, Zendura and Zendura Flex. The ones who have the highest colony countwere CWRU flex light and DX30. See Graph 1.

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Graph 1 - Graphic Adhesion of S. Mutans between the 10 different materials



Graph 2 - Box plot displaying the difference between the 10 types of thermoformed plastic materials

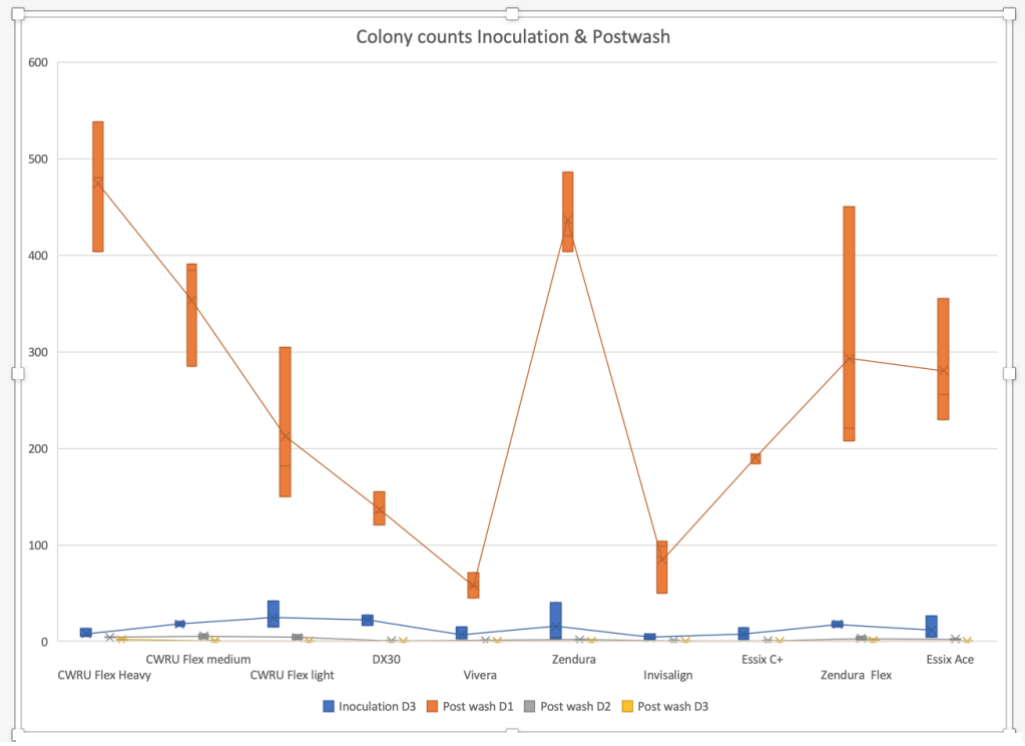
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Table 1. Mean and Standard Deviation for the 10 different type of thermoformed plastic aligners for the dilution 3 (10^9) on CFU (colony-forming unit)

Material	n	mean	SD
CWRU Flex Heavy	3	8,0	4,36
CWRU Flex medium	3	18,0	2,65
CWRU Flex light	3	24,3	15,31
DX30	3	22,7	5,13
Vivera	3	7,0	6,93
Zendura	3	15,7	21,08
Invisalign	3	4,3	3,21
Essix C+	3	7,3	6,11
Zendura Flex	3	17,7	3,06
Essix Ace	3	11,7	12,42

The water osmotic filtrated commercial water for rinsing the samples was used for 20 seconds, all colony counts decreased significantly, very low numbers, especially in the second and third dilutions. The three samples CWRU flex heavy, medium and light in the second dilution had $4x10^6$CFU, $5 x10^6$CFU and $2 x10^6$ all other samples had 1 to the 10^6 CFU. In the third dilution most sample went to zero in colony count except for CWRU flex heavy 2 to 10^9, and medium and Zendura flex had a decrease 1 to 10^9 each. See graphic 2.

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Graph2 – Colony counts Inoculation and Postwash

Table 2. Mean and Standard Deviation for the 10 different type of thermoformed plastic aligners for the dilution 1 (10^3) , 2 (10^6) and 3 (10^9)on CFU (colony-forming unit) after wash

Material	dilution					
	1st /10^3		2nd / 10^6		3rd / 10^9	
	mean	SD	mean	SD	média	dp
CWRU Flex Heavy	474.0	67.20	4.0	0.00	1.7	0.58
CWRU Flex medium	353.3	59.28	5.3	2.52	0.3	0.58
CWRU Flex light	212.3	81.83	4.0	2.65	-	-
DX30	136.7	17.16	0.3	0.58	-	-
Vivera	57.7	13.01	1.0	0.00	-	-
Zendura	436.7	43.47	1.7	0.58	-	-
Invisalign	84.3	29.84	0.7	0.58	-	-
Essix C+	190.3	5.51	0.7	0.58	-	-
Zendura Flex	293.0	136.12	3.0	2.00	0.3	0.58
Essix Ace	280.3	65.96	2.0	1.00	-	-

All quality control performed well, including purity control in each batch of Streptococcus mutans BHI and blood agar sterility plates. Also, after switching to commercial water

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sterility plate did not growth bacteria. The UV light 30 minutes upside up and 30 minutes up side up was enough to sterilize the plastic thermoformed materials for the experiments.

5. Discussion

Based on the experimental results of the present study, the null hypothesis was rejected for the bacteria adhesion since significant colonies formation were encountered in the surface of the different plastics.

In the present study, ten different thermoformed plastic materials were used for determination of the surface adherence of Streptococcus mutans. The main reasons were determining between of the most commonly used plastic material for aligners and retainers which one had an ability to grow more bacteria in an In vitro ideal condition or to see if any of those material had any antimicrobial properties. All tests were performed using brain heart infusion broth at 37^{o}C for 24 hs.

Comparing the adherence of Streptococcus mutans on ten commonly use tested thermoformed plastic materials all grew bacteria in an in vitro ideal condition simulating oral cavity brain heart infusion broth at 37for 24 hours. They testes samples displayed significant difference with the best performance on invisaling.

Rinsing thermoformed plastic material with clean water for 20 seconds significantly decreased the colony count invitro control study, implying that this will decrease the number of bacteria in the surface of the removable appliance.

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The difference present in the dilution when comparing each sample against itself (triplicates) and It was only present on the dilutions and not in the imprints, It was presume effect of the location in the molar buccal part where the sample was collected (more bacteria on some deeper molar parts than others), and these difference were less evident after water rinse. The difference disappeared on the second and third dilution after water rinse per say an effect of the a dilution.

Limited information is available to recommend patient how to clean their removable thermoformed orthodontic appliances, these results suggest rinsing with clean water will significantly decrease the amount Streptococcus mutans on the surface of the thermoformed plastic material. Likely frequent rinses will effectible and safely decrease the number of bacteria growing in the surface of the orthodontic removable appliance without affecting the physicochemical characteristic of the appliance. Timed studies for optimization of rinsing time with clean water.

In real time appliance are wear usually for shorter periods of time that varied in each patient from 2 to 12 hours and the recommended add total time in a day is 20 to 22 hours. Invitro 24 hours growth was enough time for pure Streptococcus mutans to form a biofilm on the surface of appliance without competing with other bacteria, candida ssp. Other microorganism and inhibitory condition of saliva, mastication, etc.

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6. Conclusion

The results of this study suggest that all the different plastic aligners tested do not have any antimicrobial properties that can avoid biofilm formation during the tested period of 24 hours. The thermoformed plastic that had the best performance regardless S. Mutans adherence was Invisalign aligners and the one who absorbed more was CWRU flex light followed by DX30. After wash with water we noticed a decrease in bacteria count with CWRU flex heavy the one who showed less decrease followed by CWRU flex medium and Zendura Flex.

7. References

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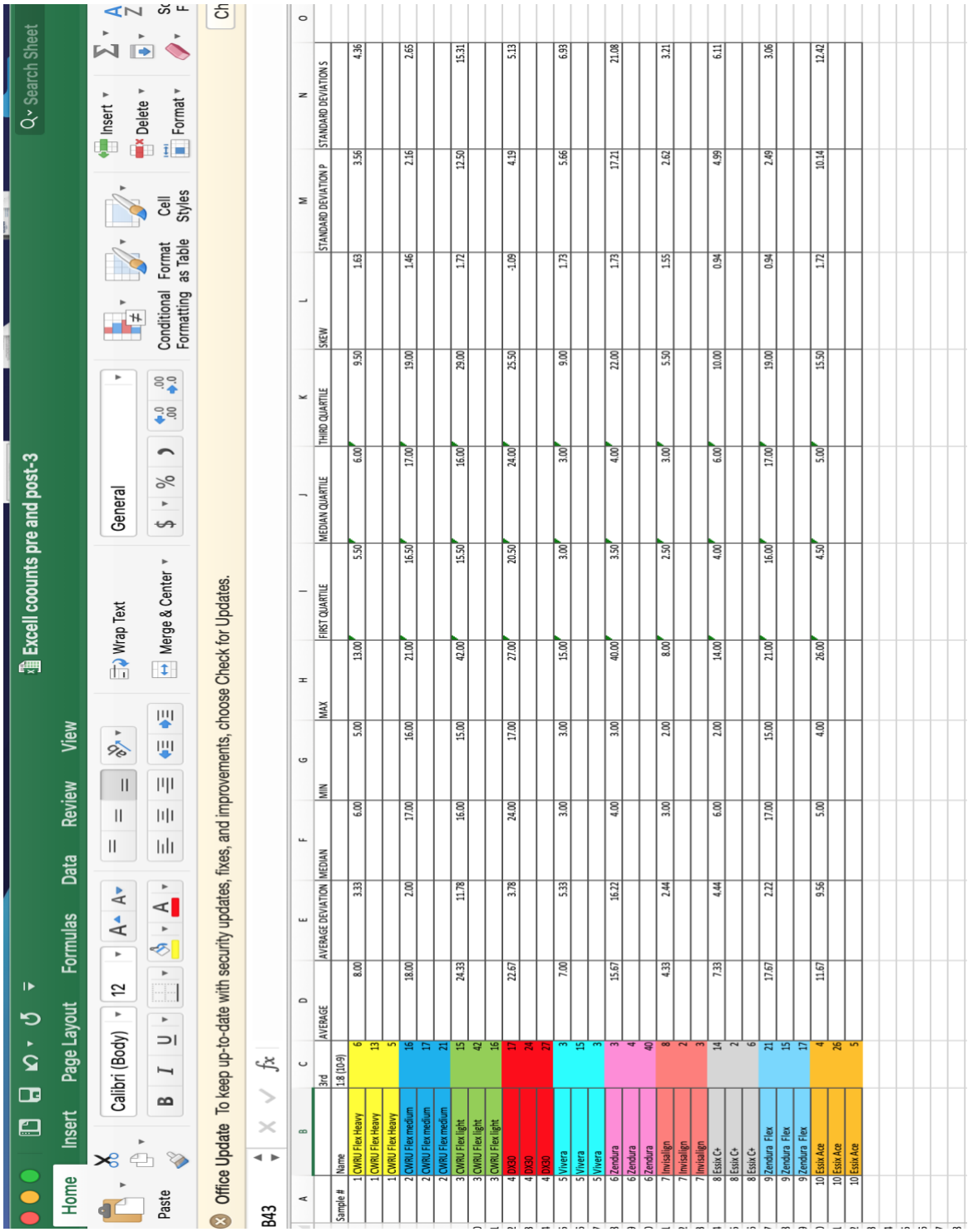
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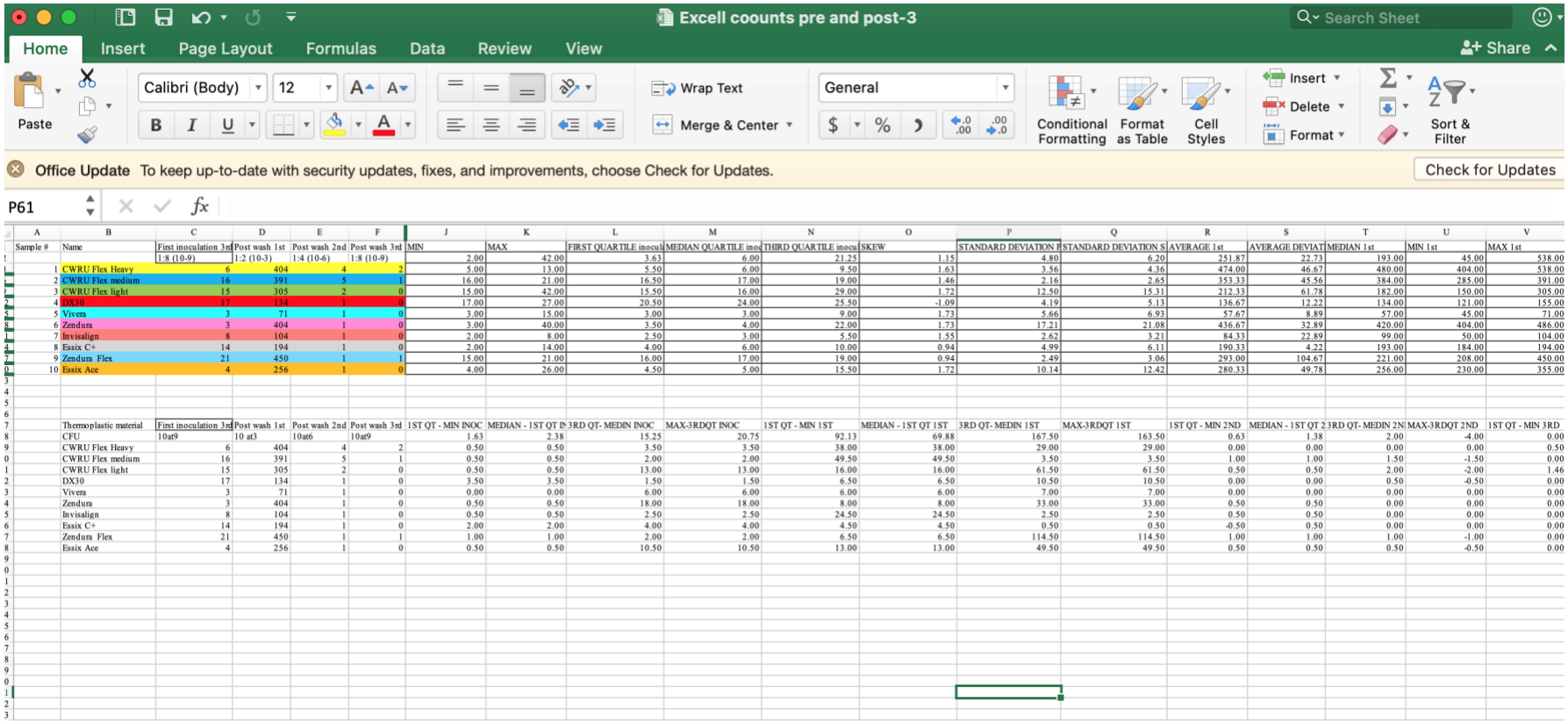
7-Appendices

7.1 - Appendice A - Aditional descriptive tables



Excell coounts pre and post-3

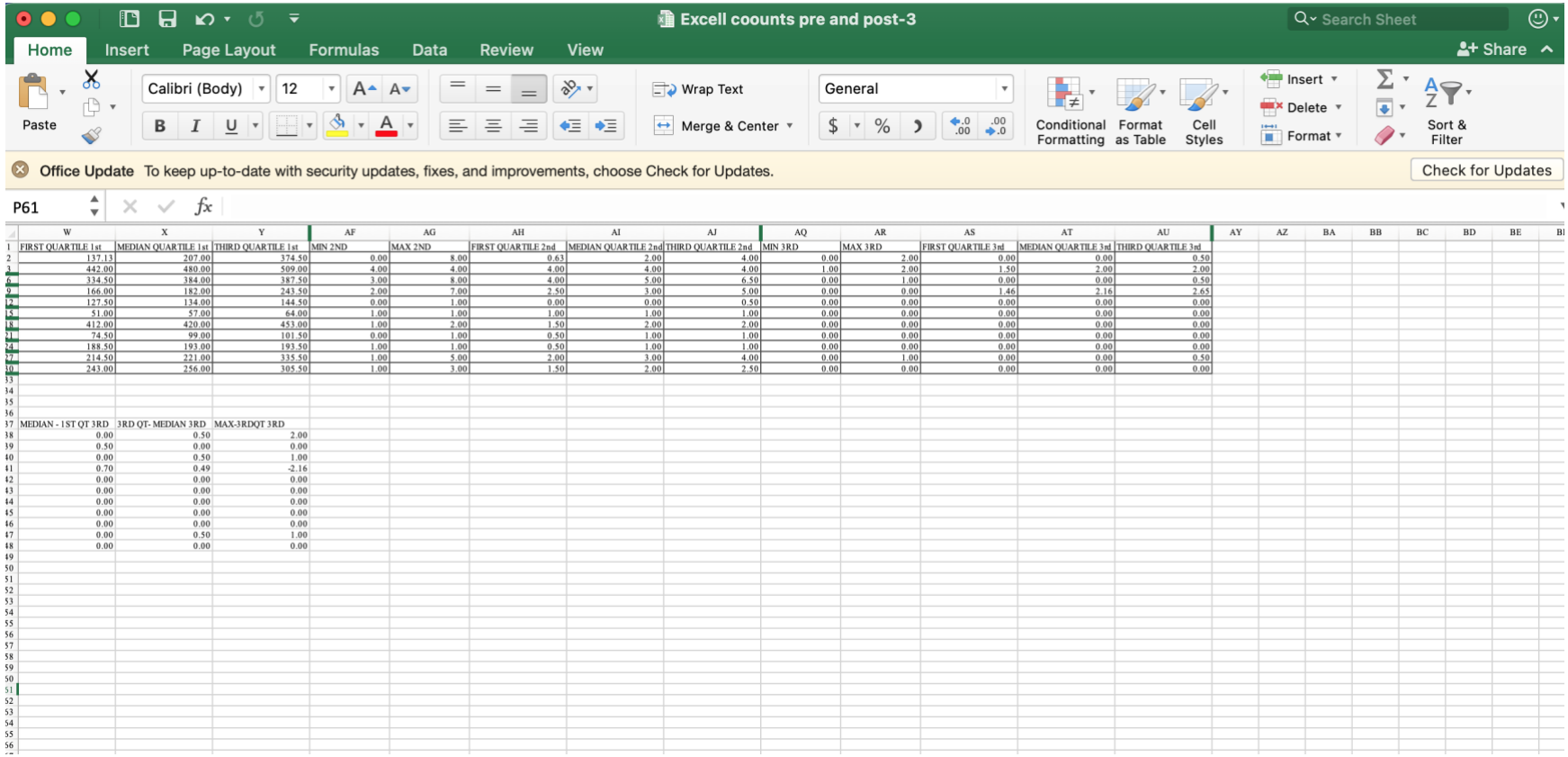
Sample #		3rd	AVERAGE	AVERAGE DEVIATION	MEDIAN	MIN	MAX	FIRST QUARTILE	MEDIAN QUARTILE	THIRD QUARTILE	SKEW	STANDARD DEVIATION P	STANDARD DEVIATION S
	Name	1:8 (10-9)											
1	CVRU Flex Heavy	6	8.00	3.33		6.00	5.00	13.00	5.50	6.00	9.50	1.63	3.56	4.36
1	CVRU Flex Heavy	13												
1	CVRU Flex Heavy	5												
2	CVRU Flex medium	16	18.00	2.00	17.00	16.00	21.00	16.50	17.00	19.00	1.46	2.16	2.65	
2	CVRU Flex medium	17												
2	CVRU Flex medium	21												
3	CVRU Flex light	15	24.33	11.78	16.00	15.00	42.00	15.50	16.00	29.00	1.72	12.50	15.31	
3	CVRU Flex light	42												
3	CVRU Flex light	16												
4	DX30	17	22.67	3.78	24.00	17.00	27.00	20.50	24.00	25.50	-1.09	4.19	5.13	
4	DX30	24												
4	DX30	27												
5	Vivera	3	7.00	5.33	3.00	3.00	15.00	3.00	3.00	9.00	1.73	5.66	6.93	
5	Vivera	15												
5	Vivera	3												
6	Zendura	3	15.67	16.22	4.00	3.00	40.00	3.50	4.00	22.00	1.73	17.21	21.08	
6	Zendura	4												
6	Zendura	40												
7	Invisalign	8	4.33	2.44	3.00	2.00	8.00	2.50	3.00	5.50	1.55	2.62	3.21	
7	Invisalign	2												
7	Invisalign	3												
8	Esix C+	14	7.33	4.44	6.00	2.00	14.00	4.00	6.00	10.00	0.94	4.99	6.11	
8	Esix C+	2												
8	Esix C+	6												
9	Zendura Flex	21	17.67	2.22	17.00	15.00	21.00	16.00	17.00	19.00	0.94	2.49	3.06	
9	Zendura Flex	15												
9	Zendura Flex	17												
10	Esix Ace	4	11.67	9.56	5.00	4.00	26.00	4.50	5.00	15.50	1.72	10.14	12.42	
10	Esix Ace	26												
10	Esix Ace	5												



Sample #	Name	First inoculation 3rd 1:8 (10-9)	Post wash 1st 1:2 (10-3)	Post wash 2nd 1:4 (10-6)	Post wash 3rd 1:8 (10-9)	MIN	MAX	FIRST QUARTILE inocul	MEDIAN QUARTILE inoc	THIRD QUARTILE inocu	SKEW	STANDARD DEVIATION F	STANDARD DEVIATION S	AVERAGE 1st	AVERAGE DEVIAT	MEDIAN 1st	MIN 1st	MAX 1st
						2.00	42.00	3.63	6.00	21.25	1.15	4.80	6.20	251.87	22.73	193.00	45.00	538.00
1	CWRU Flex Heavy	6	404	4	2	5.00	13.00	5.50	6.00	9.50	1.63	3.56	4.36	474.00	46.67	480.00	404.00	538.00
2	CWRU Flex medium	16	391	5	1	16.00	21.00	16.50	17.00	19.00	1.46	2.16	2.65	353.33	45.56	384.00	285.00	391.00
3	CWRU Flex light	15	305	2	0	15.00	42.00	15.50	16.00	29.00	1.72	12.50	15.31	212.33	61.78	182.00	150.00	305.00
4	DX30	17	134	1	0	17.00	27.00	20.50	24.00	25.50	-1.09	4.19	5.13	136.67	12.22	134.00	121.00	155.00
5	Vivera	3	71	1	0	3.00	15.00	3.00	3.00	9.00	1.73	5.66	6.93	57.67	8.89	57.00	45.00	71.00
6	Zendura	3	404	1	0	3.00	40.00	3.50	4.00	22.00	1.73	17.21	21.08	436.67	32.89	420.00	404.00	486.00
7	Invisalign	8	104	1	0	2.00	8.00	2.50	3.00	5.50	1.55	2.62	3.21	84.33	22.89	99.00	50.00	104.00
8	Essix C+	14	194	1	0	2.00	14.00	4.00	6.00	10.00	0.94	4.99	6.11	190.33	4.22	193.00	184.00	194.00
9	Zendura Flex	21	450	1	1	15.00	21.00	16.00	17.00	19.00	0.94	2.49	3.06	293.00	104.67	221.00	208.00	450.00
10	Essix Ace	4	256	1	0	4.00	26.00	4.50	5.00	15.50	1.72	10.14	12.42	280.33	49.78	256.00	230.00	355.00

	Thermoplastic material	First inoculation 3rd	Post wash 1st	Post wash 2nd	Post wash 3rd	1ST QT - MIN INOC	MEDIAN - 1ST QT IN	3RD QT- MEDIN INOC	MAX-3RDQT INOC	1ST QT - MIN 1ST	MEDIAN - 1ST QT 1ST	3RD QT- MEDIN 1ST	MAX-3RDQT 1ST	1ST QT - MIN 2ND	MEDIAN - 1ST QT 2	3RD QT- MEDIN 2N	MAX-3RDQT 2ND	1ST QT - MIN 3RD
	CFU	10at9	10 at3	10at6	10at9	1.63	2.38	15.25	20.75	92.13	69.88	167.50	163.50	0.63	1.38	2.00	-4.00	0.00
	CWRU Flex Heavy	6	404	4	2	0.50	0.50	3.50	3.50	38.00	38.00	29.00	29.00	0.00	0.00	0.00	0.00	0.50
	CWRU Flex medium	16	391	5	1	0.50	0.50	2.00	2.00	49.50	49.50	3.50	3.50	1.00	1.00	1.50	-1.50	0.00
	CWRU Flex light	15	305	2	0	0.50	0.50	13.00	13.00	16.00	16.00	61.50	61.50	0.50	0.50	2.00	-2.00	1.46
	DX30	17	134	1	0	3.50	3.50	1.50	1.50	6.50	6.50	10.50	10.50	0.00	0.00	0.50	-0.50	0.00
	Vivera	3	71	1	0	0.00	0.00	6.00	6.00	6.00	6.00	7.00	7.00	0.00	0.00	0.00	0.00	0.00
	Zendura	3	404	1	0	0.50	0.50	18.00	18.00	8.00	8.00	33.00	33.00	0.50	0.50	0.00	0.00	0.00
	Invisalign	8	104	1	0	0.50	0.50	2.50	2.50	24.50	24.50	2.50	2.50	0.50	0.50	0.00	0.00	0.00
	Essix C+	14	194	1	0	2.00	2.00	4.00	4.00	4.50	4.50	0.50	0.50	-0.50	0.50	0.00	0.00	0.00
	Zendura Flex	21	450	1	1	1.00	1.00	2.00	2.00	6.50	6.50	114.50	114.50	1.00	1.00	1.00	-1.00	0.00
	Essix Ace	4	256	1	0	0.50	0.50	10.50	10.50	13.00	13.00	49.50	49.50	0.50	0.50	0.50	-0.50	0.00

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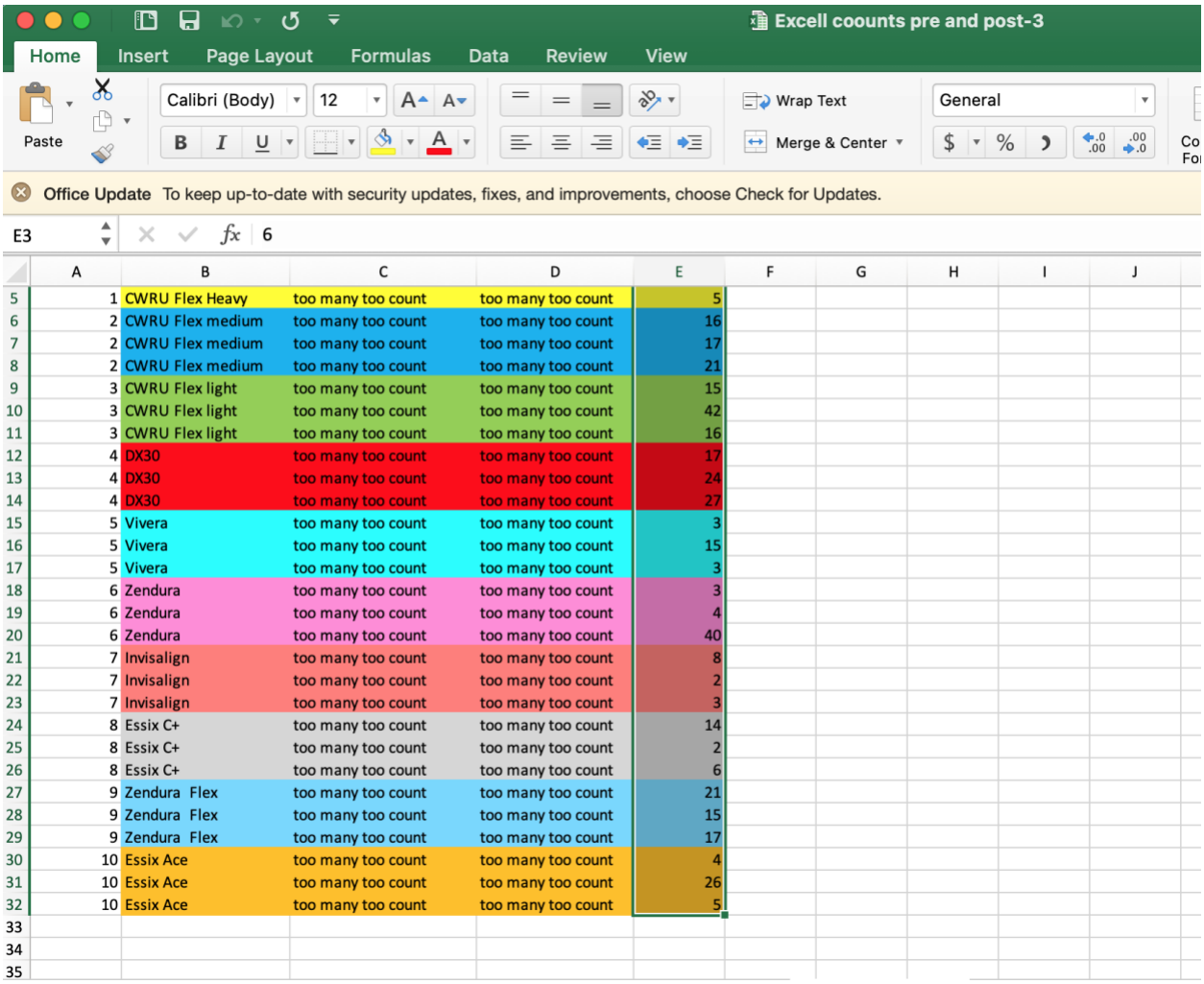
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FIRST QUARTILE 1st	MEDIAN QUARTILE 1st	THIRD QUARTILE 1st	MIN 2ND	MAX 2ND	FIRST QUARTILE 2nd	MEDIAN QUARTILE 2nd	THIRD QUARTILE 2nd	MIN 3RD	MAX 3RD	FIRST QUARTILE 3nd	MEDIAN QUARTILE 3nd	THIRD QUARTILE 3nd
137.13	207.00	374.50	0.00	8.00	0.63	2.00	4.00	0.00	2.00	0.00	0.00	0.50
442.00	480.00	509.00	4.00	4.00	4.00	4.00	4.00	1.00	2.00	1.50	2.00	2.00
334.50	384.00	387.50	3.00	8.00	4.00	5.00	6.50	0.00	1.00	0.00	0.00	0.50
166.00	182.00	243.50	2.00	7.00	2.50	3.00	5.00	0.00	0.00	1.46	2.16	2.65
127.50	134.00	144.50	0.00	1.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00
51.00	57.00	64.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00
412.00	420.00	453.00	1.00	2.00	1.50	2.00	2.00	0.00	0.00	0.00	0.00	0.00
74.50	99.00	101.50	0.00	1.00	0.50	1.00	1.00	0.00	0.00	0.00	0.00	0.00
188.50	193.00	193.50	1.00	1.00	0.50	1.00	1.00	0.00	0.00	0.00	0.00	0.00
214.50	221.00	335.50	1.00	5.00	2.00	3.00	4.00	0.00	1.00	0.00	0.00	0.50
243.00	256.00	305.50	1.00	3.00	1.50	2.00	2.50	0.00	0.00	0.00	0.00	0.00

MEDIAN - 1ST QT 3RD	3RD QT- MEDIAN 3RD	MAX-3RDQT 3RD
0.00	0.50	2.00
0.50	0.00	0.00
0.00	0.50	1.00
0.70	0.49	-2.16
0.00	0.00	0.00
0.00	0.00	0.00
0.00	0.00	0.00
0.00	0.00	0.00
0.00	0.00	0.00
0.00	0.50	1.00
0.00	0.00	0.00

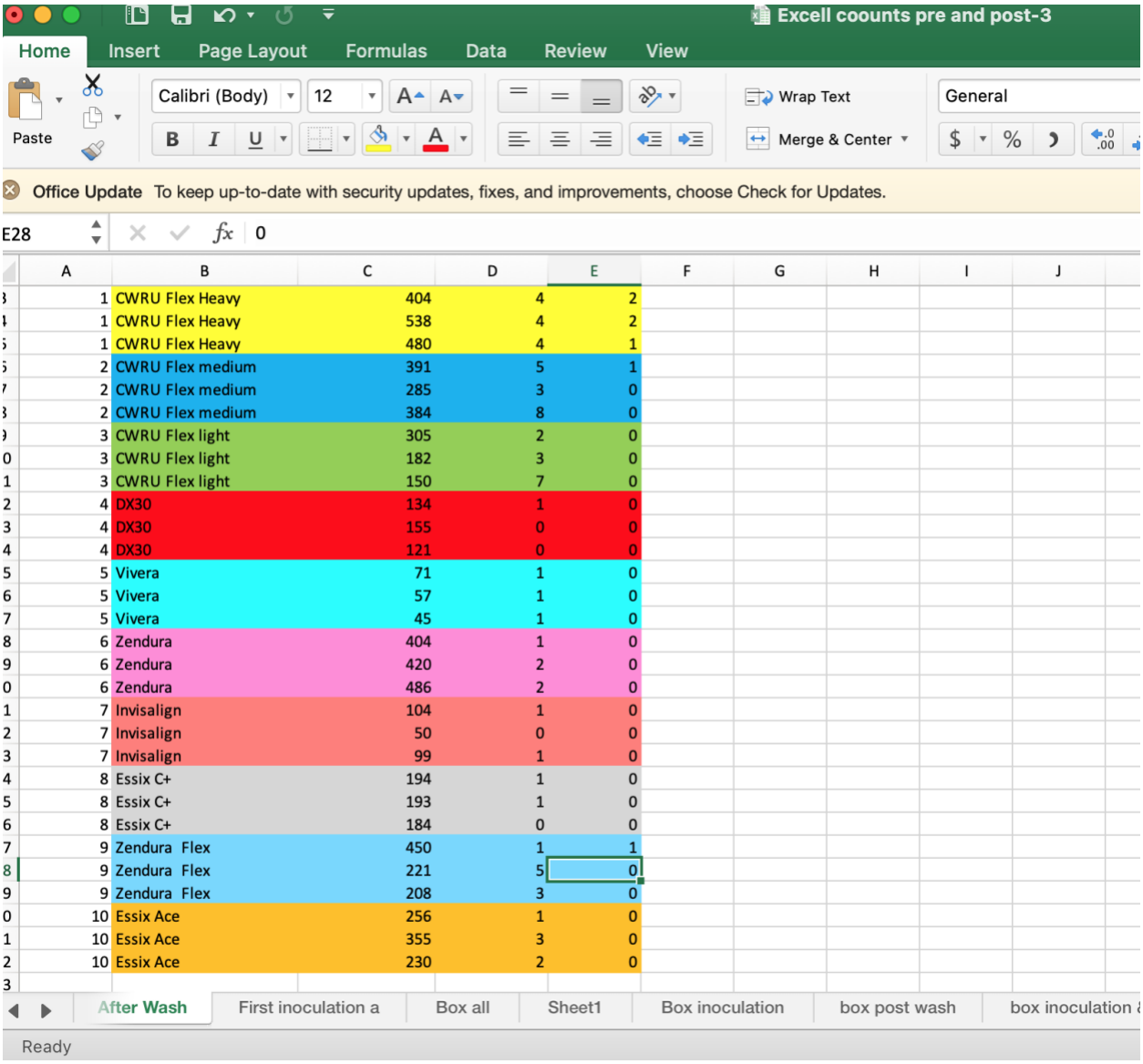
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7.2 - Appendice 2 - Raw data



	A	B	C	D	E	F	G	H	I	J
5	1	CWRU Flex Heavy	too many too count	too many too count	5					
6	2	CWRU Flex medium	too many too count	too many too count	16					
7	2	CWRU Flex medium	too many too count	too many too count	17					
8	2	CWRU Flex medium	too many too count	too many too count	21					
9	3	CWRU Flex light	too many too count	too many too count	15					
10	3	CWRU Flex light	too many too count	too many too count	42					
11	3	CWRU Flex light	too many too count	too many too count	16					
12	4	DX30	too many too count	too many too count	17					
13	4	DX30	too many too count	too many too count	24					
14	4	DX30	too many too count	too many too count	27					
15	5	Vivera	too many too count	too many too count	3					
16	5	Vivera	too many too count	too many too count	15					
17	5	Vivera	too many too count	too many too count	3					
18	6	Zendura	too many too count	too many too count	3					
19	6	Zendura	too many too count	too many too count	4					
20	6	Zendura	too many too count	too many too count	40					
21	7	Invisalign	too many too count	too many too count	8					
22	7	Invisalign	too many too count	too many too count	2					
23	7	Invisalign	too many too count	too many too count	3					
24	8	Essix C+	too many too count	too many too count	14					
25	8	Essix C+	too many too count	too many too count	2					
26	8	Essix C+	too many too count	too many too count	6					
27	9	Zendura Flex	too many too count	too many too count	21					
28	9	Zendura Flex	too many too count	too many too count	15					
29	9	Zendura Flex	too many too count	too many too count	17					
30	10	Essix Ace	too many too count	too many too count	4					
31	10	Essix Ace	too many too count	too many too count	26					
32	10	Essix Ace	too many too count	too many too count	5					
33										
34										
35										



Excell coounts pre and post-3

Home | Insert | Page Layout | Formulas | Data | Review | View

Paste | Calibri (Body) | 12 | Wrap Text | General | Merge & Center

Office Update To keep up-to-date with security updates, fixes, and improvements, choose Check for Updates.

E28 | 0

	A	B	C	D	E	F	G	H	I	J
3	1	CWRU Flex Heavy	404	4	2					
4	1	CWRU Flex Heavy	538	4	2					
5	1	CWRU Flex Heavy	480	4	1					
6	2	CWRU Flex medium	391	5	1					
7	2	CWRU Flex medium	285	3	0					
8	2	CWRU Flex medium	384	8	0					
9	3	CWRU Flex light	305	2	0					
10	3	CWRU Flex light	182	3	0					
11	3	CWRU Flex light	150	7	0					
12	4	DX30	134	1	0					
13	4	DX30	155	0	0					
14	4	DX30	121	0	0					
15	5	Vivera	71	1	0					
16	5	Vivera	57	1	0					
17	5	Vivera	45	1	0					
18	6	Zendura	404	1	0					
19	6	Zendura	420	2	0					
20	6	Zendura	486	2	0					
21	7	Invisalign	104	1	0					
22	7	Invisalign	50	0	0					
23	7	Invisalign	99	1	0					
24	8	Essix C+	194	1	0					
25	8	Essix C+	193	1	0					
26	8	Essix C+	184	0	0					
27	9	Zendura Flex	450	1	1					
28	9	Zendura Flex	221	5	0					
29	9	Zendura Flex	208	3	0					
30	10	Essix Ace	256	1	0					
31	10	Essix Ace	355	3	0					
32	10	Essix Ace	230	2	0					
33										

After Wash | First inoculation a | Box all | Sheet1 | Box inoculation | box post wash | box inoculation

Ready

DS MH DS JMP DS SG