May 2019

Prevalence of Aggregatibacter Actinomycetemcomitans and Fusobacterium Nucleatum among Clinical Orthodontic Saliva Samples

Jason Robert Klingler
jasonklingler1@gmail.com

PREVALENCE OF AGGREGATIBACTER ACTINOMYCETEMCOMITANS AND FUSOBACTERIUM NUCLEATUM AMONG CLINICAL ORTHODONTIC SALIVA SAMPLES

By

Jason Klingler

Bachelor of Business Management
Brigham Young University
2012

Doctor of Dental Medicine
University of Nevada, Las Vegas
2016

A thesis submitted in partial fulfillment
of the requirements for the

Master of Science - Oral Biology

School of Dental Medicine
Division of Health Sciences
The Graduate College

University of Nevada, Las Vegas
May 2019

UNLV | GRADUATE COLLEGE

Thesis Approval

The Graduate College
The University of Nevada, Las Vegas

February 19, 2019

This thesis prepared by

Jason Klingler

entitled

Prevalence of Aggregatibacter Actinomycetemcomitans and Fusobacterium Nucleatum among Clinical Orthodontic Saliva Samples

is approved in partial fulfillment of the requirements for the degree of

Master of Science - Oral Biology
School of Dental Medicine

Karl Kingsley, Ph.D.
Examination Committee Chair

Kathryn Hausbeck Korgan, Ph.D.
Graduate College Interim Dean

Brian Chrzan, Ph.D.
Examination Committee Member

Tanya Al-Talib, D.D.S.
Examination Committee Member

Jennifer Pharr, Ph.D.
Graduate College Faculty Representative

Abstract

Prevalence of Aggregatibacter Actinomycetemcomitans and Fusobacterium Nucleatum Among Clinical Orthodontic Saliva Samples

By

Jason Klingler

Dr. Karl Kingsley, Examination Committee Chair
Associate Professor of Biomedical Sciences
Director of Student Research
University of Nevada, Las Vegas
School of Dental Medicine

Changes to the oral microflora occur when there are shifts in the levels of the numerous bacterial species. Changes in bacterial load occur in health, disease, and dental treatments such as orthodontics and can be detected through saliva. Many studies dealing with saliva have centered around detecting bacteria known for correlation with chronic periodontitis and caries. Fewer have focused on bacterial species that contribute to microbial shifts not strictly correlated with disease. Measuring the degree of disease progression or future susceptibility is not always possible with traditional clinical parameters alone.

Aggregatibacter actinomycetemcomitans (AA) is a bacterial strain that serves as a bridging species among the oral microbiome. Although it is commonly associated with localized aggressive periodontitis, it is also found commonly in the oral flora not suffering from that

severe periodontal condition. AA plays an important role within the oral microbiome as it acts as a bridging species that allows other species of bacteria to coaggregate around it.

Altered tooth surfaces in the oral cavity, such as when fixed orthodontic appliances are in place, introduces surface area for plaque accumulation and impediments to daily plaque removal from the teeth while reducing the efficiency of natural plaque-removal mechanisms, such as salivary flow accompanied by movement of the oral mucosa and tongue. Although, some evidence exists about using unstimulated saliva as a screening tool for overtly putative species, limited evidence suggests screening for bacterial burden of bacterial species such as AA, which precede and contribute to coaggregation and heterotypic community formation.

The data from the following two studies provide evidence that salivary screening of orthodontic patients may be a non-invasive means to detect changes to important periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*. both adult and pediatric orthodontic patients have an increased prevalence of AA, compared adult and pediatric patients not currently in fixed orthodontic appliances. Additionally, the data clearly suggest a correlation between overall microbial oral burden and *Aggregatibacter* presence in orthodontic patients. Directionality of the relationship, that is whether unidirectional or bidirectional, is yet to be established. More detailed longitudinal studies on this topic could elucidate this relationship. These data provide strong evidence that more research is needed and that continued focus in this area may provide clinical guidelines for assessment of risk for patients undergoing orthodontic treatment.

Acknowledgments

Thank you to my committee chair, Dr. Karl Kingsley, for introducing me to this topic. His support and dedication of time throughout my research project has been invaluable. Also, thank you my committee members, Dr. Tanya Al-Talib, Dr. Brian Chrzan, and Dr. Jennifer Pharr for your support. I would like to thank Ching Shen for her time and help in collecting salivary samples and help in the laboratory.

Table of Contents

List of Tables

Chapter 2

Chapter 3

List of Figures

Chapter 2

Chapter 3

Chapter 1: Introduction

Background and Significance

The oral flora is a complex ecosystem characterized by numerous bacterial species and changes to the levels of these bacteria in health, disease, and dental treatments such as orthodontics. Many studies of the oral flora are centered around consensus bacteria responsible for caries and chronic periodontal disease. Other virulent bacterial strains may receive less attention because their mere presence is not well correlated with the presence of chronic periodontal disease. One of these bacterial strains is A.A. *Aggregatibacter actinomycetemcomitans* (AA) is a commensal bacterium found among the oral flora [1].

This organism is a facultative non-motile, gram negative, rod shaped bacteria. It is commonly associated with localized aggressive periodontitis but is also found commonly in the oral flora not suffering from that severe periodontal condition [6]. In addition to oral infections, its several serotypes have a variety of virulence factors enable to evade defense mechanisms of many tissues and is capable of being found in infections of the skin, GI tract, and, sinus and reproductive system [2,3,4,5,7]. Recent evidence indicates that its presence in adults is associated with risk of pre-diabetes, metabolic syndrome, and coronary artery disease [2,4,5,6].

However, little is known regarding individual differences and if orthodontic treatment will result in changes to the salivary levels of this bacterial species. Fixed orthodontic appliances introduces new surfaces for plaque accumulation and obstacles to removing daily plaque on and between teeth. The appliances reduce the efficiency of natural plaque removal mechanisms, such as salivary flow accompanied by movement of the oral mucosa and tongue. It also increases the difficulty of standard oral hygiene practices of brushing and flossing. This altered environment caused by fixed orthodontic appliances creates

Research Question

The aim of this study is to evaluate the prevalence of AA among Orthodontic and non-Orthodontic patients from a public dental school clinic.

1. Is there variation in the prevalence of AA between adult orthodontic and non-orthodontic patients?

   H_0: Microbial profiles demonstrate there is no difference in the prevalence of *Aggregatibacter actinomycetemcomitans* (AA) among orthodontic and non-orthodontic patients at UNLV SDM.

   H_A: Microbial profiles demonstrate there is an increase in the prevalence of *Aggregatibacter actinomycetemcomitans* in orthodontic patients when compared to non-orthodontic patients at UNLV SDM.

2. Is there variation int the prevalence of F.N. between adult orthodontic and non-orthodontic patients?

   H_0: Microbial profiles demonstrate there is no difference in the prevalence of *Fusobacterium nucleatum* (FN) among orthodontic and non-orthodontic patients at UNLV SDM.

   H_A: Microbial profiles demonstrate there is an increase in the prevalence of *Fusobacterium nucleatum* in orthodontic patients when compared to non-orthodontic patients at UNLV SDM.

Approval

This project was reviewed and approved by the Institutional Review Board (IRB) and Office for the Protection of Human Subjects (OPRS) at the University of Nevada, Las Vegas OPRS#1502-506M titled "The Prevalence of Oral Microbes in Saliva from the University of Nevada, Las Vegas – School of Dental Medicine pediatric and adult clinical population". Inclusion criteria included all current patients of record at UNLV-SDM clinics. Exclusion criteria included any patient who declined to participate and any subject who was not a patient of record at UNLV-SDM.

Research Design

This research design is retrospective. Using an approved sampling protocol, saliva samples were obtained from Orthodontic (n=39) and non-Orthodontic (n=45) patients. DNA was successfully isolated from 96.4% (n=81/84) patient samples. Relative endpoint polymerase chain reaction (RE-PCR) was used to subsequently screen these samples for the presence and relative abundance of AA.

References

1. Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the Normal Bacterial Flora of the Oral Cavity. Journal of Clinical Microbiology, 43(11), 5721–5732. http://doi.org/10.1128/JCM.43.11.5721-5732.2005
2. Africa, C. W. J., Nel, J., & Stemmet, M. (2014). Anaerobes and bacterial vaginosis in pregnancy: virulence factors contributing to vaginal colonisation. International Journal of Environmental Research and Public Health, 11(7), 6979–7000. http://doi.org/10.3390/ijerph110706979
3. Chen, C., Wang, T., & Chen, W. (2010). Occurrence of Aggregatibacter actinomycetemcomitans serotypes in subgingival plaque from United States subjects. Molecular Oral Microbiology, 25(3), 207–214. http://doi.org/10.1111/j.2041-1014.2010.00567.x
4. Demmer, R. T., Jacobs, D. R., Singh, R., Zuk, A., Rosenbaum, M., Papapanou, P. N., & Desvarieux, M. (2015). Periodontal Bacteria and Prediabetes Prevalence in ORIGINS: The Oral Infections, Glucose Intolerance, and Insulin Resistance Study. Journal of Dental Research, 94(9 Suppl), 201S–11S. http://doi.org/10.1177/0022034515590369
5. Mäntylä, P., Buhlin, K., Paju, S., Persson, G. R., Nieminen, M. S., Sinisalo, J., & Pussinen, P. J. (2013). Subgingival Aggregatibacter actinomycetemcomitans associates with the risk of coronary artery disease. Journal of Clinical Periodontology, 40(6), 583–590. http://doi.org/10.1111/jcpe.12098
6. Paju, S., Pussinen, P. J., Suominen-Taipale, L., Hyvönen, M., Knuuttila, M., & Könönen, E. (2009). Detection of multiple pathogenic species in saliva is associated with periodontal

infection in adults. Journal of Clinical Microbiology, 47(1), 235–8. http://doi.org/10.1128/JCM.01824-08

7. Takada, K., Saito, M., Tsuzukibashi, O., Kawashima, Y., Ishida, S., & Hirasawa, M. (2010). Characterization of a new serotype g isolate of Aggregatibacter actinomycetemcomitans. Molecular Oral Microbiology, 25(3), 200–206. http://doi.org/10.1111/j.2041-1014.2010.00572.x

8. Yáñez-Vico, R.-M., Iglesias-Linares, A., Ballesta-Mudarra, S., Ortiz-Ariza, E., Solano-Reina, E., & Perea, E.-J. (2015). Short-term effect of removal of fixed orthodontic appliances on gingival health and subgingival microbiota: A prospective cohort study. Acta Odontologica Scandinavica, 73(7), 496–502. http://doi.org/10.3109/00016357.2014.993701

Chapter 2

Prevalence of Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum among Clinical Orthodontic and Non-Orthodontic Saliva Samples

This chapter has been published in "Journal of Advances in Microbiology (JAMB)", *An Int. Journal,* and is presented in the style of that Journal. The complete Citation is:

Klingler J, Shen C, Kingsley K. Prevalence of Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum among Clinical Orthodontic and Non-Orthodontic Saliva Samples. Journal of Advances in Microbiology 11(3): 1-9, 2018; DOI: 10.9734/JAMB/2018/42698.

Role of Authors:
This work was carried out in collaboration between all authors. Authors Ching Shen and Dr. Jason Klingler were responsible for sample collection, informed consent, DNA isolation and experimental protocol. Authors Dr. Karl Kingsley and Dr. Jason Klingler were responsible for project design, funding and manuscript preparation. All authors read and approved the final manuscript.

Abstract

Objectives: The oral flora is a complex ecosystem characterized by numerous bacterial species and changes to the levels of these bacteria in health, disease, and dental treatments such as orthodontics. Although some studies have documented changes in periodontal pathogen burden during orthodontic treatment using saliva, most have focused on traditional cariogenic bacteria and some periodontal pathogens, such as *Porphyromonas gingivalis* or Fuso*bacterium nucleatum*– far fewer have focused on *Aggregatibacter actinomycetemcomitans* – commonly associated with aggressive periodontitis. Therefore, the main objective of this study was to evaluate the prevalence of this organism among Orthodontic and non-Orthodontic patients from a public dental school clinic.

Experimental Methods: Using an approved protocol, samples were taken from Orthodontic (n=39) and non-Orthodontic (n=45) age-matched patients. DNA was extracted and screened for

Aggregatibacter actinomycetemcomitans. Males and females were equally represented, although a majority of patients participating in this study were Hispanics and ethnic minorities.

Results: PCR analysis of the DNA isolated from these patient samples revealed that more than half (54%) of the Orthodontic samples harbored significant levels of *Aggregatibacter actinomycetemcomitans*, compared with only one-quarter (25%) of samples from non-Orthodontic patients. In addition, screening for *Fusobacterium nucleatum* revealed a slightly increased prevalence among Orthodontic patients (27%) compared with non-Orthodontic patients (19%).

Conclusions: These results are significant as *Aggregatibacter actinomycetemcomitans* has been traditionally observed as facilitating heterotypic communities of overtly pathogenic organisms, compared with other gram-negative oral microbes. These heterotypic biofilm communities exhibit greatly increased capacities to resist antimicrobial drugs and other host immune factors and the capacity to facilitate heterotypic associations within the biofilm may be restricted to a few key species. This project successfully demonstrated evidence that non-invasive salivary screening of orthodontic patients may be sufficient to assess and detect changes to this periodontal pathogen – thereby increasing the potential quality and efficiency of Orthodontic dental treatment among this patient population

Key Words: *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum,* saliva screening, microbial prevalence, Orthodontic treatment

Abbreviations: *Aggregatibacter actinomycetemcomitans* (AA), *Fusobacterium nucleatum* (FN), Institutional Review Board (IRB), Office for the Protection of Human Subjects (OPRS), University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM),

Polymerase chain reaction (PCR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), deoxyribonucleic acid (DNA),

1. Introduction

The oral flora is a complex ecosystem characterized by numerous bacterial species and changes to the levels of these bacteria in health, disease, and dental treatments such as orthodontics [1.2]. Many studies of the oral flora are centered around consensus bacteria responsible for caries and chronic periodontal disease [3-6]. Other virulent bacterial strains may receive less attention because their mere presence is not strictly correlated with the presence of chronic periodontal disease [7-10].

One of these bacterial strains is *Aggregatibacter actinomycetemcomitans* (AA), a commensal bacterium found among the oral flora [7,11,12]. This organism is a facultative non-motile, gram negative, bacillus commonly associated with localized aggressive periodontitis, but is also found commonly in the oral flora not suffering from that severe periodontal condition [13,14]. In addition to oral infections, its several serotypes have a variety of virulence factors enable to evade defense mechanisms of many tissues and is capable of being found in infections of the skin, GI tract, sinus and reproductive systems [15-19]. Recent evidence indicates that its presence in adults is associated with risk of pre-diabetes, metabolic syndrome, and coronary artery disease [20-23].

Although some evidence has demonstrated changes to subgingival periodontal microbes such as AA, little is known regarding whether orthodontic treatment will result in changes to the salivary levels of this bacterial species – a non-invasive and more readily assessed measure of risk [7-9,24,25]. Fixed orthodontic appliances introduces new surfaces for plaque accumulation and obstacles to removing daily plaque on and between teeth while reducing the efficiency of

natural plaque removal mechanisms, such as salivary flow accompanied by movement of the oral mucosa and tongue [26,27]. Although some studies have documented the change in periodontal pathogen burden during orthodontic treatment using saliva, most have focused on traditional cariogenic bacteria and some periodontal pathogens, such as *Porphyromonas gingivalis* - but not *Aggregatibacter* [8,28-30].

Based upon this paucity of evidence, the main objective of this study was to evaluate the prevalence of AA among Orthodontic and non-Orthodontic patients from a public dental school clinic. The main research question was to assess if there is variation in the prevalence of AA between adult orthodontic and non-orthodontic patients that is detectable in salivary samples taken from these patients. Successful completion of this project would provide preliminary evidence that non-invasive salivary screening of orthodontic patients may assess changes to this periodontal pathogen – thereby increasing the quality and efficiency of dental treatment among this patient population.

2. Methodology

2.1 Project approval

This project was reviewed and approved by the Institutional Review Board (IRB) and Office for the Protection of Human Subjects (OPRS) at the University of Nevada, Las Vegas OPRS#1502-506M titled "The Prevalence of Oral Microbes in Saliva from the University of Nevada, Las Vegas – School of Dental Medicine pediatric and adult clinical population". Inclusion criteria included all current patients of record at UNLV-SDM clinics. Exclusion criteria included any patient who declined to participate and any subject who was not a patient of record at UNLV-SDM.

2.2 Sample collection

In brief, all adult patients were asked to provide Informed Consent, while pediatric patients were asked to provide Pediatric Assent and their parent or guardian was asked to provide Parental Permission. Each sample and corresponding demographic information intake sheet was assigned a randomly generated, non-duplicated identifier that was designed to protect patient information. Demographic information included only basic information, such as Sex, Age, and Race or Ethnicity.

2.3 DNA isolation

Patient saliva samples were brought to the biomedical laboratory for storage at -80C until processing. In brief, patient samples were processed using the GenomicPrep DNA isolation kit from Amersham Biosciences (Little Chalfont, UK). Quantification and quality of DNA was assessed using spectrophotometric UV absorbance readings at 260 and 280 nm (A260, A280). DNA with a ratio of A260:A280 greater than 1.65 was subsequently screened using PCR and primers specific for *Aggregatibacter actinomycetemcomitans* (AA).

2.4 PCR screening

Polymerase Chain Reaction (PCR) screening of the isolated DNA was accomplished using the exACTGene complete PCR kit from Fisher Scientific (Fair Lawn, NJ) and an Eppendorf MasterCycler (Hamburg, Germany). A positive control for human DNA was used – glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme from the glycolytic pathway. In addition, a positive control for bacterial DNA was also used – 16S rRNA universal primer, to confirm the presence of bacterial DNA. Primers for *Aggregatibacter actinomycetemcomitans*

(AA) and *Fusobacterium nucleatum* (FN) were also synthesized by Eurofins Genomics (Louisville, KY):

GAPDH forward primer, 5'-ATC TTC CAG GAG CGA GAT CC-3'; 20 nt, 55% GC, Tm=66°C

GAPDH reverse primer, 5'-ACC ACT GAC ACG TTG GCA GT-3'; 20 nt, 55%GC, Tm=70°C

Annealing temperature: 67°C

16S rRNA universal primer, 5'-ACG CGT CGA CAG AGT TTG ATC CTG GCT-3'; 27 nt, 56% GC, Tm=76°C

16S rRNA universal primer, 5'-GGG ACT ACC AGG GTA TCT AAT-3'; 21 nt, 48% GC, Tm=62°C

Annealing temperature: 63°C

AA forward primer, 5'-ATT GGG GTT TAG CCC TGG T-3'; 19 nt, 53% GC, Tm=67°C

AA reverse primer, 5'-GGC ACA AAC CCA TCT CTG A-3'; 19 nt, 53%GC, Tm=65°C

Annealing temperature: 66°C

FN primer (forward); 5'-CGC AGA AGG TGA AAG TCC TGT AT-3'; 23 nt, 48% GC, Tm 67°C

FN primer (reverse); 5'-TGG TCC TCA CTG ATT CAC ACA GA-3'; 23 nt, 48% GC, Tm 68°C

Annealing temperature: 68°C

2.5 Statistical analysis

Using the approved sampling protocol, saliva samples were obtained from Orthodontic and non-Orthodontic patients. Simple descriptive statistics of the study sample and the clinic population were provided, and Chi Square analysis was used to determine any differences among the demographic groups (Sex, Age, Race or Ethnicity). Following PCR screening, differences between demographics of positive and negative samples also were assessed using Chi Square analysis

3. Results

A total of thirty-nine (n=39) Orthodontic samples and forty-five (n=45) non-Orthodontic samples were collected from clinic patients, yielding a total study sample size of eighty-four (n=84) (Table 1). Analysis of these demographics revealed that the percentages of females in the study samples (both Orthodontic and non-Orthodontic) was slightly greater than males (56.4%, 57.8%, respectively). This was similar to the demographic distribution of females in the Orthodontic clinic at 60.4%, and not statistically significant (p=0.4142).

An evaluation of self-reported Race/Ethnicity revealed approximately one-fourth of the study sample (both Orthodontic and non-Orthodontic) identified as White or Caucasian, which was similar to the overall percentage from the Orthodontic clinic, p=0.6532. The greatest proportion of non-White or minority patients were Hispanic in both the study samples (51.3%, 51.1%) and the Orthodontic clinic (52.3%), which was also not significantly different, p=0.6532. Finally, the proportion of patients under 18 years of age was approximately half in both the study samples (51.2%, 51.1%), which was similar to the overall percentage in the Orthodontic clinic (56.7%), p=0.2255.

Table 1. Demographic analysis of study participants

	Orthodontic sample (n=39)	Non-Orthodontic sample (n=45)	Orthodontic Clinic population (n=1,463)	Statistical analysis
Sex				
Female	56.4 % (n=22)	57.8% (n=26)	60.4% (n=884)	$\chi 2=0.667$
Male	43.6% (n=17)	42.2% (n=19)	39.6% (n=579)	d.f.=1
				$p=0.4142$
Race/Ethnicity				
White	25.6% (n=10)	24.4% (n=11)	24.7% (n=361)	$\chi 2=1.627$
Hispanic	51.3% (n=20)	51.1% (n=23)	52.3% (n=765)	d.f.=3
Black	15.4% (n=6)	13.3% (n=6)	11.8% (n=172)	$p=0.6532$
Asian	7.7% (n=3)	11.1% (n=5)	7.9% (n=117)	
Other			3.3% (n=10)	
Age				
Under <18 yrs.	51.2% (n=20)	51.1% (n=23)	56.7% (n=830)	$\chi 2=1.469$
Over > 18 yrs.	48.7% (n=19)	48.9% (n=23)	43.3% (n=633)	d.f.=1
				$p=0.2255$

Each saliva sample was processed to isolate DNA, both bacterial and human (Table 2). In total, DNA was successfully isolated from n=81/84 samples (96.4%), which is well within the expected recovery range (95-100%). The average concentration of DNA from the Orthodontic samples was 699.1 ng/uL that ranged between 550 – 885 ng/uL, which is lower but comparable to the average of the non-Orthodontic samples of 804.7 ng/uL that ranged between 571 – 980 ng/uL, $p<0.01$.

Table 2. DNA isolation and analysis

	DNA analysis	Statistical analysis
Orthodontic samples (n=39)		
DNA concentration	ave.= 699.1 ng/uL	Students t-test
DNA concentration	range=550-885 ng/uL	(two-tailed)
		$p<0.01$
Non-Orthodontic samples (n=45)		
DNA concentration	ave.= 804.7 ng/uL	
DNA concentration	range=571-980 ng/uL	

The DNA from each sample was then screened using PCR for the presence of *Aggregatibacter actinomycetemcomitans* or AA (Figure 1). These results revealed that more than half of the Orthodontic samples (56.4%) had significant and detectable levels of AA, compared

with only 25% of the non-Orthodontic samples. Correspondingly, less than half of age-matched Orthodontic samples tested negative for AA, while three-quarters (75%) of the non-Orthodontic samples were found to have no AA above the threshold limit of detection.

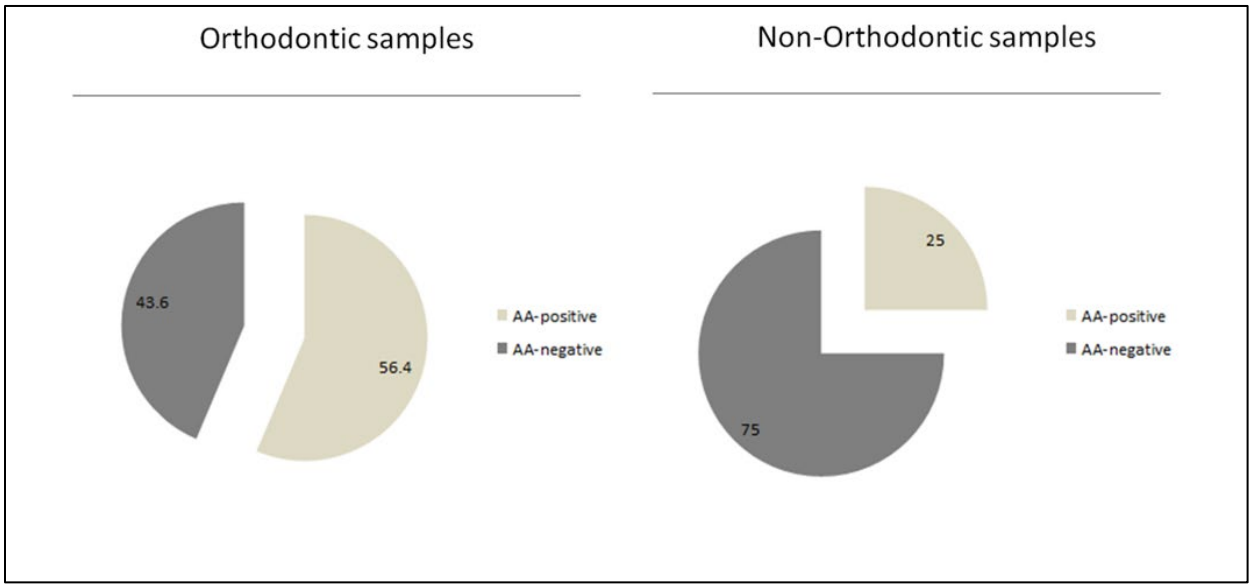

Figure 1. PCR screening of DNA isolates. PCR screening revealed 56.4% of Orthodontic samples harbored significant levels of *Aggregatibacter actinomycetemcomitans* (AA), compared with only 25% of non-Orthodontic samples. This was statistically significant, $p<0.05$.

To determine if this phenomenon was restricted to AA, another gram-negative organism was selected for screening – *Fusobacterium nucleatum* or FN (Figure 2). PCR screening of the DNA isolated from the Orthodontic and non-Orthodontic samples revealed significant levels of FN (above the limit of detection) in one fourth (27.7%) of the Orthodontic saliva samples and only one-fifth (19%) of non-Orthodontic samples tested, which was also statistically significant, $p<0.05$.

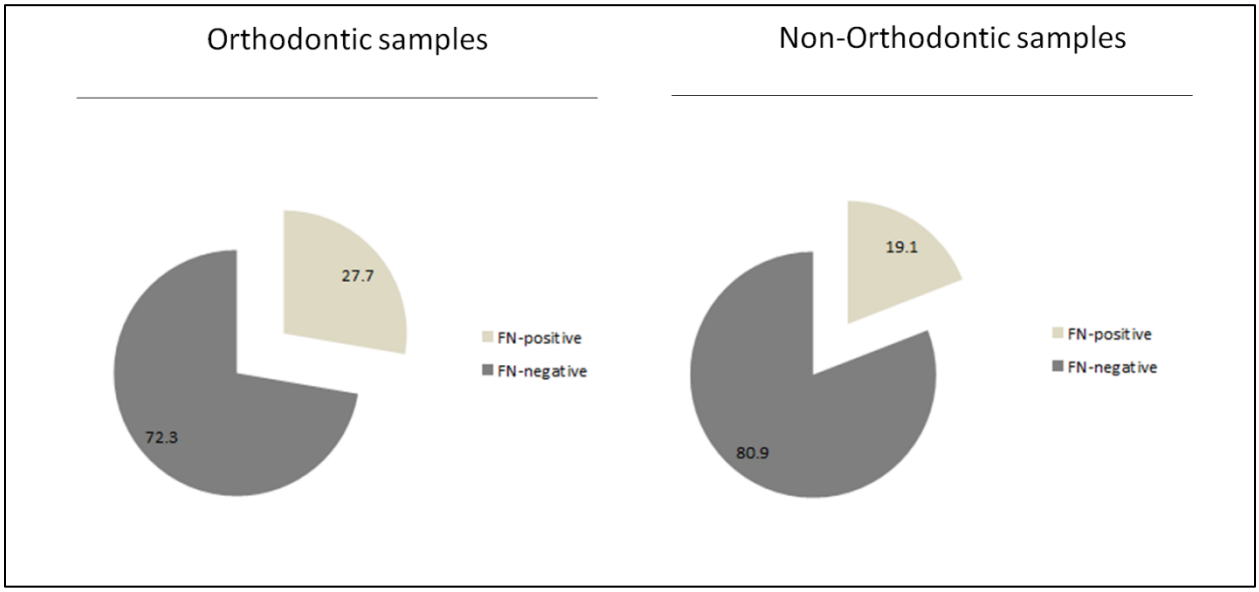

Figure 2. PCR screening of DNA isolates. PCR screening revealed 27.7% of Orthodontic samples harbored significant levels of *Fusobacterium nucleatum* (FN), compared with only 19.1% of non-Orthodontic samples. This was statistically significant, $p<0.05$.

4. Discussion

The main objective of this study was to evaluate the prevalence of *Aggregatibacter actinomycetemcomitans* or AA among Orthodontic and non-Orthodontic patients from a public dental school clinic. The results of this study demonstrate that AA is detectable in saliva samples from these patients. Moreover, the main finding was that more than half of the Orthodontic subjects harbored significant levels of AA in unstimulated saliva, compared with only one-fourth of the non-Orthodontic subjects. These results are significant as AA is mainly associated with localized aggressive periodontitis and chronic periodontitis [31,32].

These results are significant as AA has been traditionally observed as facilitating heterotypic communities of overtly pathogenic organisms, compared with other gram-negative oral microbes [33,34]. In fact, biofilm communities exhibit greatly increased capacities to resist antimicrobial drugs and other host immune factors [35,36]. The capacity to facilitate heterotypic associations within the biofilm may be restricted to a few key species, including AA [37,38].

For comparison, another gram-negative, periodontal pathogen was assessed in this study – *Fusobacterium nucleatum* or FN [39]. Although the results of this study demonstrated a difference between the prevalence of FN among Orthodontic samples (27%) compared with non-Orthodontic samples (19%), these differences were less dramatic and are more likely a secondary result due to the primary influx of AA among the Orthodontic patients [7,24]. Although these results are significant and may provide some useful biometric indicators for non-invasive biofilm community assessment among Orthodontic patients, there are some limitations associated with this type of study.

First, only non-invasively collected saliva was available for this study, which may limit the conclusions that can be made from these analyses. No corresponding direct biofilm collection was possible, therefore only inferential analyses can be made from these results. Second, and more importantly, this was a cross-sectional study that collected saliva from Orthodontic and non-Orthodontic patients at a single time point, which means no temporal information can be evaluated regarding the change in microbial prevalence over time.

5. Conclusions

Despite these limitations, this project successfully demonstrated preliminary evidence that non-invasive salivary screening of orthodontic patients may be sufficient to assess and detect

changes to periodontal pathogens, such as AA and FN – thereby increasing the potential quality and efficiency of Orthodontic dental treatment among this patient population.

6. Acknowledgements

The authors would like to thank the Department of Advanced Education Program in Orthodontics and Dentofacial Orthopedics, as well as Dr. Jeffrey Ebersole and the Office of Research at the University of Nevada, Las Vegas – School of Dental Medicine for funding to support this project. The authors would also like to thank Steven Lam for his assistance in the collection and organization of clinical samples.

7. Competing Interests

The authors have declared that no competing interests exist.

8. Author Contributions

Ching Shen and Dr. Jason Klingler were responsible for sample collection, informed consent, DNA isolation and experimental protocol. Dr. Karl Kingsley and Dr. Jason Klingler were responsible for project design, funding and manuscript preparation.

References

1. Gao L, Xu T, Huang G, Jiang S, Gu Y, Chen F. Oral microbiomes: more and more importance in oral cavity and whole body. Protein Cell. 2018 May 7. doi: 10.1007/s13238-018-0548-1. [Epub ahead of print] Review. PMID: 29736705
2. Zhang Y, Wang X, Li H, Ni C, Du Z, Yan F. Human oral microbiota and its modulation for oral health. Biomed Pharmacother. 2018 Mar; 99:883-893. doi: 10.1016/j.biopha.2018.01.146. Epub 2018 Feb 20. Review. PMID: 29710488
3. Sudhakara P, Gupta A, Bhardwaj A, Wilson A. Oral Dysbiotic Communities and Their Implications in Systemic Diseases. Dent J (Basel). 2018 Apr 16;6(2). pii: E10. doi: 10.3390/dj6020010. Review. PMID: 29659479
4. Bowen WH, Burne RA, Wu H, Koo H. Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments. Trends Microbiol. 2018 Mar;26(3):229-242. doi: 10.1016/j.tim.2017.09.008. Epub 2017 Oct 30. Review. PMID: 29097091
5. Sampaio-Maia B, Caldas IM, Pereira ML, Pérez-Mongiovi D, Araujo R. The Oral Microbiome in Health and Its Implication in Oral and Systemic Diseases. Adv Appl Microbiol. 2016; 97:171-210. doi: 10.1016/bs.aambs.2016.08.002. Epub 2016 Sep 21. Review. PMID: 27926431
6. Tanner AC, Kressirer CA, Faller LL. Understanding Caries from the Oral Microbiome Perspective. J Calif Dent Assoc. 2016 Jul;44(7):437-46. Review. PMID: 27514155
7. Guo R, Lin Y, Zheng Y, Li W. The microbial changes in subgingival plaques of orthodontic patients: a systematic review and meta-analysis of clinical trials. BMC Oral Health. 2017 Jun 2;17(1):90. doi: 10.1186/s12903-017-0378-1. PMID: 28576147

8. Kim K, Jung WS, Cho S, Ahn SJ. Changes in salivary periodontal pathogens after orthodontic treatment: An in vivo prospective study. Angle Orthod. 2016 Nov;86(6):998-1003. Epub 2015 Nov 25. PMID: 27792427

9. Jung WS, Kim K, Cho S, Ahn SJ. Adhesion of periodontal pathogens to self-ligating orthodontic brackets: An in-vivo prospective study. Am J Orthod Dentofacial Orthop. 2016 Sep;150(3):467-75. doi: 10.1016/j.ajodo.2016.02.023. PMID: 27585775

10. Freitas AO, Marquezan M, Nojima Mda C, Alviano DS, Maia LC. The influence of orthodontic fixed appliances on the oral microbiota: a systematic review. Dental Press J Orthod. 2014 Mar-Apr;19(2):46-55. Review. PMID: 24945514

11. Gholizadeh P, Pormohammad A, Eslami H, Shokouhi B, Fakhrzadeh V, Kafil HS. Oral pathogenesis of Aggregatibacter actinomycetemcomitans. Microb Pathog. 2017 Dec; 113:303-311. doi: 10.1016/j.micpath.2017.11.001. Epub 2017 Nov 5. Review. PMID: 29117508

12. Periasamy S, Kolenbrander PE. Aggregatibacter actinomycetemcomitans builds mutualistic biofilm communities with Fusobacterium nucleatum and Veillonella species in saliva. Infect Immun. 2009 Sep;77(9):3542-51. doi: 10.1128/IAI.00345-09. Epub 2009 Jun 29. PMID: 19564387

13. Shahabuddin N, Boesze-Battaglia K, Lally ET. Trends in Susceptibility to Aggressive Periodontal Disease. Int J Dent Oral Health. 2016;2(4). doi: 10.16966/2378-7090.197. Epub 2016 Apr 25. PMID: 28008419

14. Nibali L. Aggressive Periodontitis: microbes and host response, who to blame? Virulence. 2015;6(3):223-8. doi: 10.4161/21505594.2014.986407. Review. PMID: 25654663

15. Moazzam AA, Rajagopal SM, Sedghizadeh PP, Zada G, Habibian M. Intracranial bacterial infections of oral origin. J Clin Neurosci. 2015 May;22(5):800-6. doi: 10.1016/j.jocn.2014.11.015. Epub 2015 Mar 21. Review. PMID: 25800939

16. Shilo S, Kassis I, Hakim F, Shachor-Meyouhas Y. Aggregatibacter actinomycemcomitans pneumonia in children: two case reports and a review of the literature. Pediatr Infect Dis J. 2015 Jan;34(1):100-2. doi: 10.1097/INF.0000000000000493. Review. PMID: 25068288

17. Rahamat-Langendoen JC, van Vonderen MG, Engström LJ, Manson WL, van Winkelhoff AJ, Mooi-Kokenberg EA. Brain abscess associated with Aggregatibacter actinomycetemcomitans: case report and review of literature. J Clin Periodontol. 2011 Aug;38(8):702-6. doi: 10.1111/j.1600-051X.2011.01737. x. Epub 2011 May 3. Review. PMID: 21539594

18. Demmer RT, Desvarieux M. Periodontal infections and cardiovascular disease: the heart of the matter. J Am Dent Assoc. 2006 Oct;137 Suppl:14S-20S; quiz 38S. Review. Erratum in: J Am Dent Assoc. 2008 Mar;139(3):252. PMID: 17012731

19. Fine DH, Kaplan JB, Kachlany SC, Schreiner HC. How we got attached to Actinobacillus actinomycetemcomitans: A model for infectious diseases. Periodontol 2000. 2006; 42:114-57. Review. PMID: 16930309

20. Singh-Hüsgen P, Meissner T, Bizhang M, Henrich B, Raab WH. Investigation of the oral status and microorganisms in children with phenylketonuria and type 1 diabetes. Clin Oral Investig. 2016 May;20(4):841-7. doi: 10.1007/s00784-015-1564-7. Epub 2015 Aug 23. PMID: 26297129

21. Demmer RT, Jacobs DR Jr, Singh R, Zuk A, Rosenbaum M, Papapanou PN, Desvarieux M. Periodontal Bacteria and Prediabetes Prevalence in ORIGINS: The Oral Infections, Glucose Intolerance, and Insulin Resistance Study. J Dent Res. 2015 Sep;94(9 Suppl):201S-11S. doi: 10.1177/0022034515590369. Epub 2015 Jun 16. PMID: 26082387

22. Pietiäinen M, Kopra KAE, Vuorenkoski J, Salminen A, Paju S, Mäntylä P, Buhlin K, Liljestrand JM, Nieminen MS, Sinisalo J, Hyvärinen K, Pussinen PJ. Aggregatibacter actinomycetemcomitans serotypes associate with periodontal and coronary artery disease status. J Clin Periodontol. 2018 Apr;45(4):413-421. doi: 10.1111/jcpe.12873. Epub 2018 Mar 15. PMID: 29385645

23. Liljestrand JM, Paju S, Pietiäinen M, Buhlin K, Persson GR, Nieminen MS, Sinisalo J, Mäntylä P, Pussinen PJ. Immunologic burden links periodontitis to acute coronary syndrome. Atherosclerosis. 2018 Jan; 268:177-184. doi: 10.1016/j.atherosclerosis.2017.12.007. Epub 2017 Dec 6. PMID: 29232563

24. An JS, Kim K, Cho S, Lim BS, Ahn SJ. Compositional differences in multi-species biofilms formed on various orthodontic adhesives. Eur J Orthod. 2017 Oct 1;39(5):528-533. doi: 10.1093/ejo/cjw089. PMID: 28339597

25. Passariello C, Gigola P. Adhesion and biofilm formation by periodontopathogenic bacteria on different commercial brackets. Eur J Paediatr Dent. 2013 Sep;14(3):199-203. PMID: 24295004

26. Cardoso AA, Lopes LM, Rodrigues LP, Teixeira JJ, Steiner-Oliveira C, Nobre-Dos-Santos M. Influence of salivary parameters in the caries development in orthodontic patients-an

observational clinical study. Int J Paediatr Dent. 2017 Nov;27(6):540-550. doi: 10.1111/ipd.12295. Epub 2017 Mar 1. PMID: 28247450

27. Arab S, Nouhzadeh Malekshah S, Abouei Mehrizi E, Ebrahimi Khanghah A, Naseh R, Imani MM. Effect of Fixed Orthodontic Treatment on Salivary Flow, pH and Microbial Count. J Dent (Tehran). 2016 Jan;13(1):18-22. PMID: 27536324

28. Davis JE, Freel N, Findley A, Tomlin K, Howard KM, Seran CC, Cruz P, Kingsley K. A molecular survey of S. mutans and P. gingivalis oral microbial burden in human saliva using relative endpoint polymerase chain reaction (RE-PCR) within the population of a Nevada dental school revealed disparities among minorities. BMC Oral Health. 2012 Aug 27; 12:34. doi: 10.1186/1472-6831-12-34.PMID: 22925755

29. Lara-Carrillo E, Montiel-Bastida NM, Sánchez-Pérez L, Alanís-Tavira J. Effect of orthodontic treatment on saliva, plaque and the levels of Streptococcus mutans and Lactobacillus. Med Oral Patol Oral Cir Bucal. 2010 Nov 1;15(6): e924-9. PMID: 20383105

30. Kitada K, de Toledo A, Oho T. Increase in detectable opportunistic bacteria in the oral cavity of orthodontic patients. Int J Dent Hyg. 2009 May;7(2):121-5. doi: 10.1111/j.1601-5037.2008.00333.x. PMID: 19416094

31. Herbert BA, Novince CM, Kirkwood KL. Aggregatibacter actinomycetemcomitans, a potent immunoregulator of the periodontal host defense system and alveolar bone homeostasis. Mol Oral Microbiol. 2016 Jun;31(3):207-27. doi: 10.1111/omi.12119. Epub 2015 Sep 22. Review. PMID: 26197893

32. Henderson B, Nair SP, Ward JM, Wilson M. Molecular pathogenicity of the oral opportunistic pathogen Actinobacillus actinomycetemcomitans. Annu Rev Microbiol. 2003; 57:29-55. Review. PMID: 14527274

33. Whitmore SE, Lamont RJ. The pathogenic persona of community-associated oral streptococci. Mol Microbiol. 2011 Jul;81(2):305-14. doi: 10.1111/j.1365-2958.2011.07707.x. Epub 2011 Jun 3. Review. PMID: 21635580

34. Wright CJ, Burns LH, Jack AA, Back CR, Dutton LC, Nobbs AH, Lamont RJ, Jenkinson HF. Microbial interactions in building of communities. Mol Oral Microbiol. 2013 Apr;28(2):83-101. doi: 10.1111/omi.12012. Epub 2012 Dec 17. Review. PMID: 23253299

35. Koo H, Allan RN, Howlin RP, Stoodley P, Hall-Stoodley L. Targeting microbial biofilms: current and prospective therapeutic strategies. Nat Rev Microbiol. 2017 Dec;15(12):740-755. doi: 10.1038/nrmicro.2017.99. Epub 2017 Sep 25. Review. PMID: 28944770

36. Sanz M, Beighton D, Curtis MA, Cury JA, Dige I, Dommisch H, Ellwood R, Giacaman RA, Herrera D, Herzberg MC, Könönen E, Marsh PD, Meyle J, Mira A, Molina A, Mombelli A, Quirynen M, Reynolds EC, Shapira L, Zaura E. Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and periodontal diseases. Consensus report of group 1 of the Joint EFP/ORCA workshop on the boundaries between caries and periodontal disease. J Clin Periodontol. 2017 Mar;44 Suppl 18:S5-S11. doi: 10.1111/jcpe.12682. PMID: 28266109

37. Marsh PD, Zaura E. Dental biofilm: ecological interactions in health and disease. J Clin Periodontol. 2017 Mar;44 Suppl 18:S12-S22. doi: 10.1111/jcpe.12679. PMID: 28266111

38. Peyyala R, Ebersole JL. Multispecies biofilms and host responses: "discriminating the trees from the forest". Cytokine. 2013 Jan;61(1):15-25. doi: 10.1016/j.cyto.2012.10.006. Epub 2012 Nov 6. Review. PMID: 23141757

39. Jolley D, Wonder K, Chang E, Kingsley K. Oral microbial prevalence of periodontal pathogens among orthodontic patients. International Journal of Dentistry and Oral Health (IJDOH) 2016, 1(6): doi http://dx.doi.org/10.16966/2378-7090.159

Chapter 3

Aggregatibacter actinomycetemcomitans and *Fusobacterium nucleatum* prevalence correlates with salivary microbial burden in Orthodontic patients.

This chapter has been accepted for publication in the journal *International Journal of Dental Research and Reviews (IJDRR)* and is presented in the style of that Journal. The complete Citation will be:

Klingler J, Kingsley K. Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum prevalence correlates with salivary microbial burden in Orthodontic patients. International Journal of Dental Research and Reviews

Role of Authors:
This work was carried out in collaboration between all authors. Dr. Jason Klingler was responsible for the design of the study, data generation, collection, and analysis. Dr. Karl Kingsley assisted Dr. Klingler with data generation, and analysis.

Abstract

Objectives: Many factors influence the presence and growth of oral microbial flora, including the use of orthodontic appliances. Although much research has focused on classical oral pathogens, much less information is available to determine the relationship between *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* among these patients. The primary objective of this study was to determine the relationship between oral prevalence of *Aggregatibacter* and *Fusobacterium* among orthodontic and non-orthodontic patient saliva samples.

Experimental Methods: This study was a retrospective study of previously collected saliva samples from orthodontic (n=55) and non-orthodontic (n=55) patients using an approved protocol. DNA was extracted and screened for *Aggregatibacter actinomycetemcomitans* and

Fusobacterium nucleatum. Males and females were equally represented, although a majority of patients participating in this study were Hispanics and ethnic minorities.

Results: PCR analysis of the DNA revealed that 54.5% of orthodontic samples harbored significant levels of *Aggregatibacter Actinomycetemcomitans,* while 29.1% of non-orthodontic samples harbored significant levels of *Aggregatibacter Actinomycetemcomitans* (p=0.0068). In addition, screening for *Fusobacterium* revealed 38% of orthodontic samples harbored this organism, compared with 33% of non-orthodontic samples (p=0.4599). Screening of these samples using the 16S universal primer revealed AA-positive orthodontic samples had the highest PCR band intensity, with similar band intensity of AA-Negative orthodontic samples AA-positive non-orthodontic samples, AA-negative non-orthodontic samples. While screening for *Fusobacterium* using the 16S universal primer revealed higher band intensity (microbial burden) among the FN-positive samples among both the orthodontic and non-orthodontic samples. In brief, although microbial burden was lower among the corresponding non-orthodontic samples in general, the FN-positive samples were found to harbor the highest band intensity and microbial burden.

Conclusions: This study provides significant data that clearly suggest a correlation between overall microbial oral burden and *Aggregatibacter* presence in orthodontic patients. Both AA and FN were more prevalent among orthodontic patient samples than non-orthodontic samples, although the difference in the prevalence of FN was not statistically significant. In addition, it was demonstrated the AA was more prevalent than FN overall, and among each of the categories evaluated (orthodontic, non-orthodontic). AA appears to be more prevalent among patients with orthodontic brackets than FN, although both organisms appear to have similar characteristics. This may suggest that although both organisms facilitate heterotypic associations

between varying species of oral bacteria, AA may be an earlier or more significant organism in this process in orthodontic patients.

Key words: *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, Orthodontics, Salivary screening

Introduction

Many factors influence the presence and growth of oral microbial flora, including the use of orthodontic appliances [1,2]. Many studies have evaluated different methods for reducing the overall microbial burden among this patient population, with a specific focus on cariogenic and periodontal-related bacteria [3,4]. Although much research has focused on classical oral pathogens, such as *Streptococcus mutans* and *Lactobacillus acidophilus*, much less information is available to determine the relationship between *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* among these patients [5,6].

More specifically, some previous studies have demonstrated that the presence of *Aggregatibacter* (but not *Fusobacterium*) was more prevalent in the saliva of orthodontic patients [7,8]. Although many studies have evaluated the role of *Aggregatibacter* in oral pathogenesis, more efforts have recently focused on the role of this organism to influence and modulate oral ecology [9-11].

A recent review has suggested that changes in *Streptococcus* or *Aggregatibacter* prevalence among the oral microbial flora may be related to the growth and complexity of the oral bacterial community in orthodontic patients [12]. However, direct evidence of this type of relationship between oral microbial species and the effects on microbial burden remain unresolved [13,14].

Based upon this information, the objective of this study was to determine the relationship between oral prevalence of *Aggregatibacter* and *Fusobacterium* among orthodontic and non-orthodontic patient saliva samples.

Material and Methods

Protocol and approval

This study was a retrospective study of previously collected saliva samples that were originally collected under a protocol that was approved by the Institutional Review Board (IRB) and Office for the Protection of Human Subjects (OPRS) at the University of Nevada, Las Vegas OPRS#1502-506M titled "The Prevalence of Oral Microbes in Saliva from the University of Nevada, Las Vegas – School of Dental Medicine pediatric and adult clinical population". Inclusion criteria included current patients of record at the University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM) orthodontic and main patient clinics. Exclusion criteria included any patients that declined to participate in the study and any patients not being treated at the UNLV-SDM clinics.

DNA isolation

All previously collected saliva samples had DNA extracted using the GenomicPrep DNA isolation kit (Amersham Biosciences), as previously described [6,8]. The quantity and purity of the extracted DNA was determined using UV absorbance readings at 280 and 260 nm, as previously described [13,14]. Samples deemed acceptable for this study had a minimum DNA concentration of 100 ng/uL and purity (A260:A280 ratio) of 1.65 or higher.

PCR screening

DNA was screened using polymerase chain reaction (PCR) using the exACTGene complete PCR kit (Fisher Scientific) and a thermocycler (Eppendorf), as previously described [15]. To verify the presence of control (human) DNA, a positive control was used - glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme from the glycolytic pathway. Primers for bacterial DNA, including 16S rRNA universal primer, *Aggregatibacter actinomycetemcomitans* (AA), and *Fusobacterium nucleatum* (FN) were synthesized by Eurofins Genomics:

GAPDH forward primer, 5'-ATC TTC CAG GAG CGA GAT CC-3'; 20 nt, 55% GC, Tm=66°C
GAPDH reverse primer, 5'-ACC ACT GAC ACG TTG GCA GT-3'; 20 nt, 55%GC, Tm=70°C
Annealing temperature: 67°C

16S rRNA universal primer, 5'-ACG CGT CGA CAG AGT TTG ATC CTG GCT-3'; 27 nt, 56% GC, Tm=76°C
16S rRNA universal primer, 5'-GGG ACT ACC AGG GTA TCT AAT-3'; 21 nt, 48% GC, Tm=62°C
Annealing temperature: 63°C

AA forward primer, 5'-ATT GGG GTT TAG CCC TGG T-3'; 19 nt, 53% GC, Tm=67°C
AA reverse primer, 5'-GGC ACA AAC CCA TCT CTG A-3'; 19 nt, 53%GC, Tm=65°C
Annealing temperature: 66°C

FN primer (forward); 5'-CGC AGA AGG TGA AAG TCC TGT AT-3'; 23 nt, 48% GC, Tm 67°C

FN primer (reverse); 5'-TGG TCC TCA CTG ATT CAC ACA GA-3'; 23 nt, 48% GC, Tm 68°C

Annealing temperature: 68°C

Statistical analysis

Basic average statistics were compiled for the DNA parameters associated with these samples and compared using two-tailed Students t-tests. Demographic analysis was facilitated using Chi Square, which was used to determine any demographic differences among the orthodontic and non-orthodontic groups.

Results

A total of one hundred ten (n=110) patient saliva samples were identified with sufficient DNA (>100 ng/uL) and purity (A260:S280 ratio) for inclusion in this study (Table 1). More specifically fifty-five (n=55) samples from non-orthodontic patients were identified, with an average DNA concentration of 712.3 ng/uL and purity of 1.69. These samples were matched with orthodontic patient samples, with an average DNA concentration of 722.1 ng/uL and a purity of 1.71. No significant differences were found between the average DNA concentrations in each group (p=0.742).

Table 1. DNA analysis of selected samples.

	DNA analysis	Statistical analysis
Non-orthodontic samples	(n=55)	
DNA concentration	Average = 712.3 ng/uL	
DNA concentration	Range (223.1 – 1411 ng/uL)	
DNA purity	(A260:A280) average = 1.69	Students t-test
		(two-tailed)
Orthodontic samples	(n=55)	*p*=0.742
DNA concentration	Average = 722.1 ng/uL	
DNA concentration	Range (199.4 – 998.2 ng/uL)	
DNA purity	(A260:A280) average = 1.71	

The demographic analysis of these patients revealed a nearly equal distribution of males and females within each sample (Table 2). The majority of patients from each sample were Hispanic, which reflects the overall patient population of UNLV-SDM [16]. No significant differences were identified between these two samples (orthodontic, non-orthodontic) in either sex or racial/ethnic background.

Table 2. Demographic analysis of study sample population.

	Non-Orthodontic	Orthodontic	Statistical analysis
Sex			
Male	N=27 (49.1%)	N=26 (47.3%)	$\chi 2$=1.300, d.f.=1
Female	N=28 (50.9%)	N=29 (52.7%)	p=0.2543

Race/Ethnicity			
White	N=14 (25.4%)	N=15 (27.3%)	$\chi 2$=1.819 d.f.=1
Hispanic	N=30 (54.5%)	N=31 (56.4%)	p=0.1774
Black	N=6 (10.9%)	N=5 (9.1%)	
Asian/Other	N=5 (9.1%)	N=4 (7.3%)	

All samples were then screened for the presence of AA using primers specific for this organism (Figure 1). These data revealed that more than half (54.5%) of orthodontic samples harbored significant levels of AA. In contrast, approximately one third of non-orthodontic samples (29.1%) harbored significant levels of AA (*p*=0.0068).

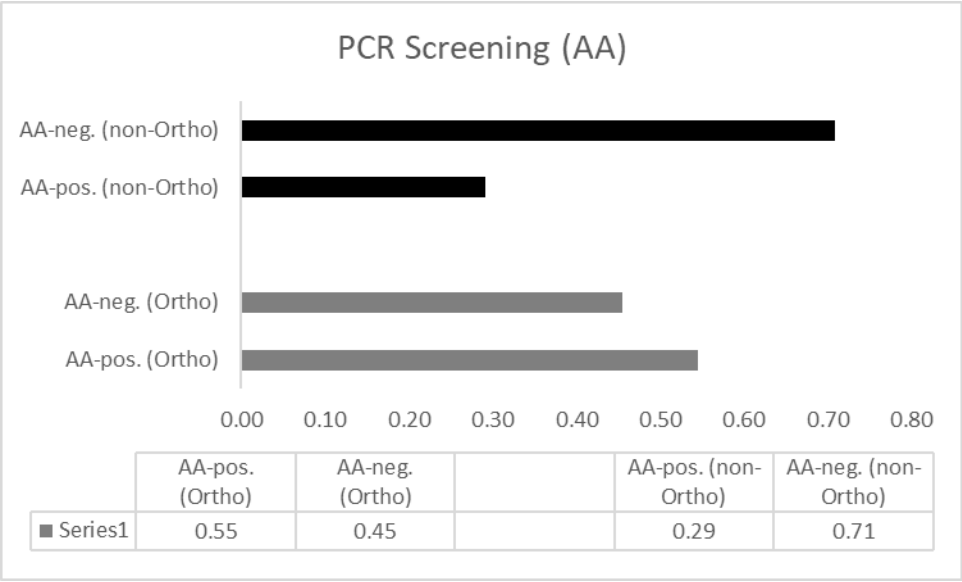

Figure 1. PCR screening of samples for AA. Salivary DNA samples screened for the presence of *Aggregatibacter* (AA) using PCR revealed 54.5% of orthodontic samples harbored this organism, compared with 29.1% of non-orthodontic samples (*p*=0.0068).

Screening of these samples using the 16S universal primer revealed the PCR band intensity was highest among the orthodontic samples which harbored AA (Figure 2). Although the PCR band intensity was higher among AA-positive than AA-negative non-orthodontic samples, these were comparable levels to the AA-negative orthodontic samples and significantly lower than the levels observed among the AA-positive orthodontic samples.

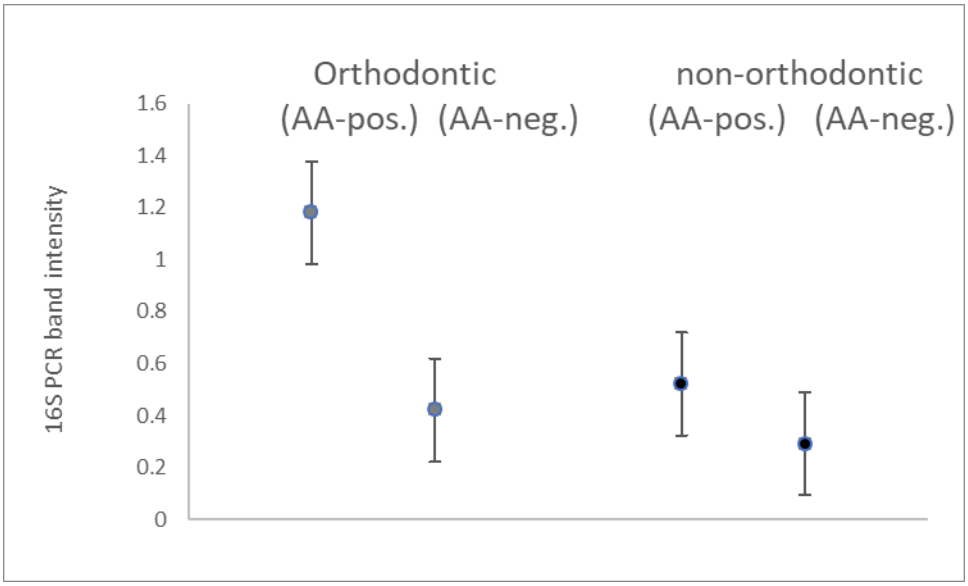

Figure 2. 16S universal primer PCR screening. Screening of samples using 16S rRNA universal primer revealed significantly higher band intensity (corresponding with bacterial levels) among the AA-positive orthodontic samples. In addition, 16S PCR band intensity was higher among AA-positive non-orthodontic samples but were significantly lower than observed among the AA-positive orthodontic samples.

Each of the samples were also screened for the presence of FN using primers specific for this organism (Figure 3). The analysis of these data revealed that slightly more than one third

(38%) of orthodontic samples harbored significant levels of FN. Among non-orthodontic samples, approximately one third (33%) were positive for FN (p=0.4599).

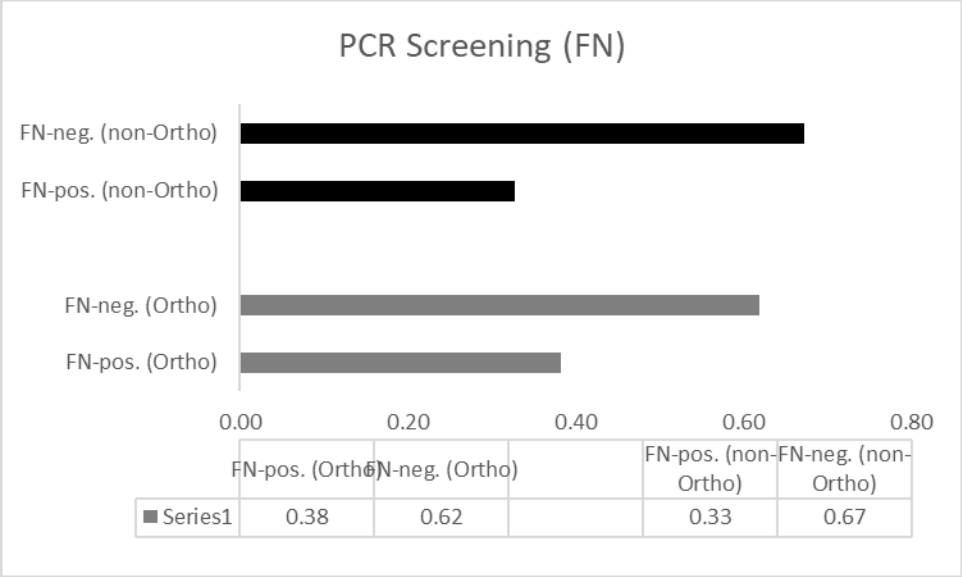

Figure 3. PCR screening of samples for FN. Salivary DNA samples screened for the presence of *Fusobacterium* (FN) using PCR revealed 38% of orthodontic samples harbored this organism, compared with 33% of non-orthodontic samples.

Analysis of these samples into the categories of FN-positive and FN-negative using the 16S universal primer revealed higher band intensity (microbial burden) among the FN-positive samples among both the orthodontic and non-orthodontic samples (Figure 4). In brief, although microbial burden was lower among the corresponding non-orthodontic samples in general, the FN-positive samples were found to harbor the highest band intensity and microbial burden.

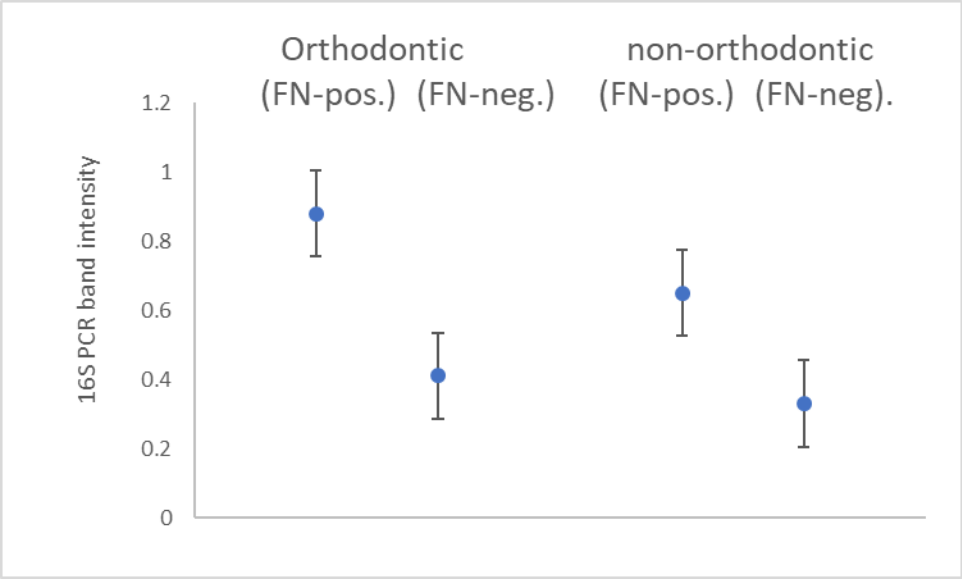

Figure 4. 16S universal primer PCR screening. Screening of samples using 16S rRNA universal primer revealed significantly higher band intensity (corresponding with bacterial levels) among the FN-positive orthodontic samples. In addition, 16S PCR band intensity was higher among FN-positive non-orthodontic samples but were significantly lower than observed among the corresponding FN-positive orthodontic samples.

Representative gel images were taken from four select patient samples to demonstrate the differences in PCR band signal intensity (Figure 5). These data demonstrated the range of signal band intensities, which ranged from low (Sample 1) to very high (Sample 4). Corresponding PCR screening for AA revealed three positives (Samples 2 – 4), while FN screening revealed only a single positive (Sample 1).

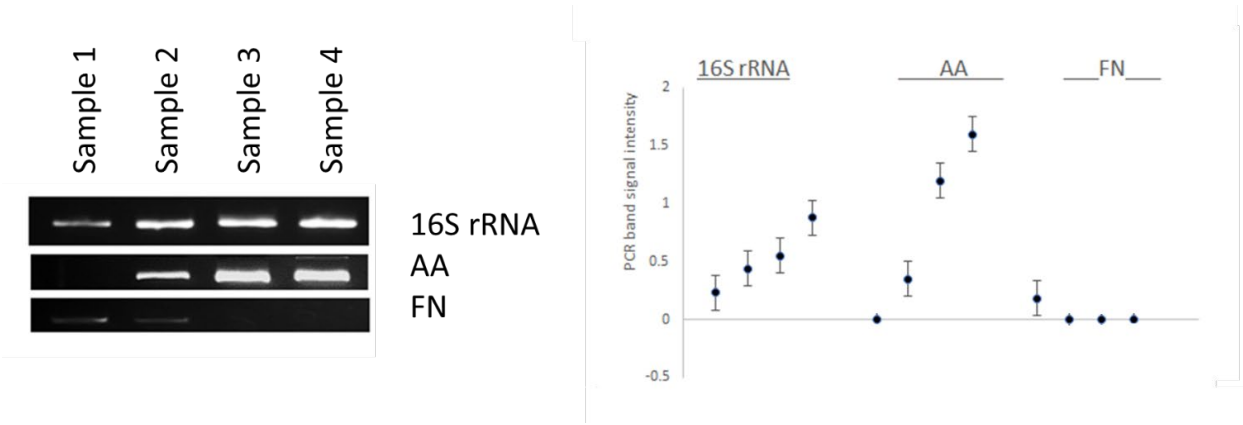

Figure 5. PCR signal band intensity. Screening of samples using PCR revealed not only the presence or absence of a particular microbial constituent, but also the relative microbial burden may be assessed using the signal band intensity of the 16S rRNA. The range of signal band intensities are shown from low (Sample 1) to high (Sample 4).

Discussion

The primary objective of this study was to determine the relationship between oral prevalence of *Aggregatibacter* and *Fusobacterium* among orthodontic and non-orthodontic patient saliva samples. These data revealed that AA was more prevalent among orthodontic patient samples than non-orthodontic samples. Although the prevalence of FN was slightly higher among orthodontic patient samples than non-orthodontic samples, this difference was not statistically significant. In addition, it was demonstrated the AA was more prevalent than FN overall, and among each of the categories evaluated (orthodontic, non-orthodontic).

These data support previous observations from this clinical population, which demonstrated orthodontic patients were more likely than non-orthodontic patients to harbor one of these organisms in significant numbers [6,8]. These observations are also supported by

clinical studies and systematic review that confirm the effects of orthodontic treatment may trigger significant changes in the composition of subgingival microbes, including AA and FN [16-18].

One significant finding is that AA appears to be more prevalent among patients with orthodontic brackets than FN, although both organisms appear to have similar characteristics [19,20]. This may suggest that although both organisms facilitate heterotypic associations between varying species of oral bacteria, AA may be an earlier or more significant organism in this process in orthodontic patients [21].

This study had intrinsic limitations that must also be considered when reviewing these findings. For example, no temporal data was available to the study authors – which may limit the inferences about microbial composition changes that might be drawn from these analyses [22,23]. In addition, due to the study limitations (time and financial) only AA and FN were evaluated for this project although many other organisms may contribute to the overall microbial composition and sub-population prevalence [24].

Conclusions

Despite these limitations, this study provides significant data that clearly suggest a correlation between overall microbial oral burden and *Aggregatibacter* presence in orthodontic patients. Whether this relationship is unidirectional or bidirectional could not be established without more detailed longitudinal studies. These data provide strong evidence that more research is needed and that continued focus in this area may provide clinical guidelines for assessment of risk for patients undergoing orthodontic treatment.

References

1. Andrucioli MC, Faria G, Nelson-Filho P, Romano FL, Matsumoto MA. Influence of resin-modified glass ionomer and topical fluoride on levels of Streptococcus mutans in saliva and biofilm adjacent to metallic brackets. J Appl Oral Sci. 2017 Mar-Apr;25(2):196-202. doi: 10.1590/1678-77572016-0231. PMID: 28403360
2. El-Patal MA, Asiry MA, AlShahrani I, El Bayoumy SY, Ahmed Wakwak MA, Mohamed Khalil MA. The effect of fiber-reinforced composite versus band and loop space maintainers on oral Lactobacillus acidophilus and Streptococcus mutans levels in saliva. J Indian Soc Pedod Prev Dent. 2018 Jul-Sep;36(3):301-307. doi: 10.4103/JISPPD.JISPPD_155_18. PMID: 30246754
3. Alp S, Baka ZM. Effects of probiotics on salivary Streptecoccus mutans and Lactobacillus levels in orthodontic patients. Am J Orthod Dentofacial Orthop. 2018 Oct;154(4):517-523. doi: 10.1016/j.ajodo.2018.01.010. PMID: 30268262
4. Aydinbelge M, Cantekin K, Herdem G, Simsek H, Percin D, Parkan OM. Changes in periodontal and microbial parameters after the space maintainers application. Niger J Clin Pract. 2017 Sep;20(9):1195-1200. doi: 10.4103/1119-3077.180070. PMID: 29072246
5. Davis JE, Freel N, Findley A, Tomlin K, Howard KM, Seran CC, Cruz P, Kingsley K. A molecular survey of S. mutans and P. gingivalis oral microbial burden in human saliva using relative endpoint polymerase chain reaction (RE-PCR) within the population of a Nevada dental school revealed disparities among minorities. BMC Oral Health. 2012 Aug 27; 12:34. doi: 10.1186/1472-6831-12-34. PMID: 22925755

6. Jolley D, Wonder K, Chang E, Kingsley K. Oral microbial prevalence of periodontal pathogens among orthodontic patients. International Journal of Dentistry and Oral Health (IJDOH) 2016, 1(6): doi http://dx.doi.org/10.16966/2378-7090.159

7. Nelson-Filho P, Carpio-Horta KO, Andrucioli MC, Feres M, Bezerra da Silva RA, Garcia Paula-Silva FW, Romano FL. Molecular detection of Aggregatibacter actinomycetemcomitans on metallic brackets by the checkerboard DNA-DNA hybridization technique. Am J Orthod Dentofacial Orthop. 2012 Oct;142(4):481-6. doi: 10.1016/j.ajodo.2012.04.021. PMID: 22999671

8. Klingler J, Shen C, Kingsley K. Prevalence of Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum among Clinical Orthodontic and Non-Orthodontic Saliva Samples. Journal of Advances in Microbiology 2018, 11(3): 1-9, 2018; DOI: 10.9734/JAMB/2018/42698

9. Gholizadeh P, Pormohammad A, Eslami H, Shokouhi B, Fakhrzadeh V, Kafil HS. Oral pathogenesis of Aggregatibacter actinomycetemcomitans. Microb Pathog. 2017 Dec; 113:303-311. doi: 10.1016/j.micpath.2017.11.001. Epub 2017 Nov 5. Review. PMID: 29117508

10. Whitmore SE, Lamont RJ. The pathogenic persona of community-associated oral streptococci. Mol Microbiol. 2011 Jul;81(2):305-14. doi: 10.1111/j.1365-2958.2011.07707.x. Epub 2011 Jun 3. Review. PMID: 21635580

11. Tsai CC, Ho YP, Chou YS, Ho KY, Wu YM, Lin YC. Aggregatibacter (Actinobacillus) actimycetemcomitans leukotoxin and human periodontitis - A historic review with emphasis on JP2. Kaohsiung J Med Sci. 2018 Apr;34(4):186-193. doi: 10.1016/j.kjms.2018.01.014. Epub 2018 Feb 17. Review. PMID: 29655406

12. Lucchese A, Bondemark L, Marcolina M, Manuelli M. Changes in oral microbiota due to orthodontic appliances: a systematic review. J Oral Microbiol. 2018 Jul 3;10(1):1476645. doi: 10.1080/20002297.2018.1476645. eCollection 2018. Review. PMID: 29988826

13. McDaniel S, McDaniel J, Tam A, Kingsley K. Howard KM. Oral Microbial Ecology of Selenemonas noxia and Scardovia wiggsiae. Microbiology Research Journal International 2017, 21(3) 1-8. DOI: 10.9734/MRJI/2017/36110

14. McDaniel J, McDaniel S, Tam A, Kingsley K, Howard KM. Screening a Saliva Repository for Scardovia wiggsiae and Streptococcus mutans: A Pilot Study. Journal of Advances in Microbiology 2017, (5) 1: 1-8. doi: 10.9734/JAMB/2017/36111

15. Quan K, Kingsley K. Effect of dental sealants on oral microbial burden of Scardovia wiggsiae within a pediatric population. A pilot study. Microbiology Research Journal International. 2018, 24(6) 1-10. DOI: 10.9734/MRJI/2018/42947

16. Papageorgiou SN, Xavier GM, Cobourne MT, Eliades T. Effect of orthodontic treatment on the subgingival microbiota: A systematic review and meta-analysis. Orthod Craniofac Res. 2018 Nov;21(4):175-185. doi: 10.1111/ocr.12237. Epub 2018 Jul 20. Review. PMID: 30028077

17. Guo R, Lin Y, Zheng Y, Li W. The microbial changes in subgingival plaques of orthodontic patients: a systematic review and meta-analysis of clinical trials. BMC Oral Health. 2017 Jun 2;17(1):90. doi: 10.1186/s12903-017-0378-1. Review. PMID: 28576147

18. An JS, Kim K, Cho S, Lim BS, Ahn SJ. Compositional differences in multi-species biofilms formed on various orthodontic adhesives. Eur J Orthod. 2017 Oct 1;39(5):528-533. doi: 10.1093/ejo/cjw089. PMID: 28339597

19. Bao K, Bostanci N, Thurnheer T, Grossmann J, Wolski WE, Thay B, Belibasakis GN, Oscarsson J. Aggregatibacter actinomycetemcomitans H-NS promotes biofilm formation and alters protein dynamics of other species within a polymicrobial oral biofilm. NPJ Biofilms Microbiomes. 2018 May 22; 4:12. doi: 10.1038/s41522-018-0055-4. eCollection 2018. PMID: 29844920

20. Wong BK, McGregor NR, Butt HL, Knight R, Liu LY, Darby IB. Association of clinical parameters with periodontal bacterial haemolytic activity. J Clin Periodontol. 2016 Jun;43(6):503-11. doi: 10.1111/jcpe.12554. Epub 2016 May 4. PMID: 27105613

21. Paolantonio M, Festa F, di Placido G, D'Attilio M, Catamo G, Piccolomini R. Site-specific subgingival colonization by Actinobacillus actinomycetemcomitans in orthodontic patients. Am J Orthod Dentofacial Orthop. 1999 Apr;115(4):423-8. PMID: 10194288

22. de Freitas AO, Alviano CS, Alviano DS, Siqueira JF Jr, Nojima LI, Nojima Mda C. Microbial colonization in orthodontic mini-implants. Braz Dent J. 2012;23(4):422-7. PMID: 23207860

23. Kim SH, Choi DS, Jang I, Cha BK, Jost-Brinkmann PG, Song JS. Microbiologic changes in subgingival plaque before and during the early period of orthodontic treatment. Angle Orthod. 2012 Mar;82(2):254-60. doi: 10.2319/030311-156.1. Epub 2011 Aug 9. PMID: 21827233

24. Verrusio C, Iorio-Siciliano V, Blasi A, Leuci S, Adamo D, Nicolò M. The effect of orthodontic treatment on periodontal tissue inflammation: A systematic review. Quintessence Int. 2018;49(1):69-77. doi: 10.3290/j.qi.a39225. Review. PMID: 29114647

Chapter 4: Summary and Conclusions.

The purpose of this project was to evaluate the prevalence of AA and FN among orthodontic and non-orthodontic patients from a public dental school clinic. Since AA and FN play an important role in forming heterotypic associations between varying species of oral bacteria, it was important to determine if non-invasive salivary screenings would be sufficient to better assess a patient's risk for an adverse microbial shift

Chapter 2 of this document was a retrospective study which used PCR analysis of DNA isolated orthodontic (n=39) and non-orthodontic (n=45) patient saliva samples screening for AA and FN. The results showed more than half (54%) of orthodontic patient samples harbored significant levels of AA in contrast to only one-quarter (25%) of non-orthodontic patient samples While screening for FN, again orthodontic patient samples had slightly increased prevalence (27%) compared with non-orthodontic patient samples (19%).

Chapter 3 was a study with a larger number of salivary samples previously collected from orthodontic (n=55) and non-orthodontic (n=55) that correlates the prevalence of AA and FN with the overall microbial burden. Again, PCR analysis of isolated DNA screened for AA among samples with 54.5% of orthodontic samples harboring significant levels of AA, compared to only 29.1% of non-orthodontic samples. Orthodontics samples screened for FN revealed 38% of samples harbored significant levels of the organism, contrasted with 33% of non-orthodontic samples. In addition, samples were screened with 16S universal primer revealing higher band intensity (microbial burden) among AA-positive orthodontic samples. While screening for FN using 16S universal primer, higher band intensity among FN-positive samples among both the orthodontic and non-orthodontic samples. This may suggest that although both organisms facilitate heterotypic associations between varying species of oral bacteria, AA may be an earlier or more significant organism in this process in orthodontic patients.

Conclusions from both chapters 2 and 3 demonstrated statistically significant differences in the prevalence of AA between Orthodontic and non-orthodontic patient samples. For the prevalence of FN, there was not a statistically significant difference in the prevalence of FN between orthodontic and non-orthodontic patients, although there may be clinical significance as both studies did demonstrate slightly higher prevalence. Based on the information presented from this study, the main question posed in this study would result in accepting the alternative hypothesis, while the secondary question would result in failing to reject the null hypothesis.

1. Is there variation in the prevalence of AA between adult orthodontic and non-orthodontic patients?

   H_A: Microbial profiles demonstrate there is an increase in the prevalence of *Aggregatibacter actinomycetemcomitans* in orthodontic patients when compared to non-orthodontic patients at UNLV SDM.

2. Is there variation int the prevalence of F.N. between adult orthodontic and non-orthodontic patients?

   H_0: Microbial profiles demonstrate there is no difference in the prevalence of *Fusobacterium nucleatum* (FN) among orthodontic and non-orthodontic patients at UNLV SDM.

Limitations and Recommendations:

As mentioned in the chapters above, one limitation of this study is that the cross-sectional nature of the study may limit inferences about changes to the microbial composition over time in orthodontic treatment. I would recommend examining longitudinal data from similar patients to

help identify additional dynamics of the patient microbial profile. Additional screening for systemic health, medications, and even dental history, such as DMFT, could be valuable. Also, because these samples were made up of strictly non-invasive saliva samples, coupling this data with other surfaces like gingival crevicular fluid, dental plaque on tooth surfaces, or buccal mucosa samples could help provide reinforcing patterns that would clarify risk indicators for adverse microbial shifts.

In addition, constraints of time and finances limited evaluation of the samples to only AA and FN, though many other organisms may contribute to the overall microbial composition. Compiling screening data for other organisms may be instructive. Most importantly, the retrospective design may limit potential patient pools that can be analyzed. This patient pool was collected within a public dental school clinic as a convenience sample, which may not represent a typical orthodontic patient pool.

Appendix A

Biomedical IRB
Notice of Excluded Activity

DATE: February 6, 2015

TO: **Dr. Karl Kingsley,** School of Dental Medicine

FROM: Office of Research Integrity – Human Subjects

RE: Notification of IRB Action
Protocol Title: **The Prevalence of Oral Microbes in Saliva from the UNLV School of Dental Medicine Pediatric and Adult Clinical Population**
Protocol# 1502-5068M

This memorandum is notification that the project referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The protocol has been reviewed and deemed excluded from IRB review. It is not in need of further review or approval by the IRB.

Any changes to the excluded activity may cause this project to require a different level of IRB review. Should any changes need to be made, please submit a Modification Form.

If you have questions or require any assistance, please contact the Office of Research Integrity – Human Subjects at IRB@unlv.edu or call 702-895-2794.

Office of Research Integrity – Human Subjects
4505 Maryland Parkway • Box 451047 • Las Vegas, Nevada 89154-1047
(702) 895-2794 • FAX: (702) 895-0805 • IRB@unlv.edu

Appendix B

Permission to Use Copyrighted Material

University of Nevada, Las Vegas

I, **Karl Kingsley,** holder of copyrighted material entitled **Prevalence of Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum among Clinical Orthodontic and Non-Orthodontic Saliva Samples,** authored by **Jason Klingler, Ching Shen,** and **Karl Kingsley** originally published in **Journal of Advances in Microbiology. July 2018, 11(3)** hereby give permission for the author to use the above described material in total or in part for inclusion in a master's thesis at the University of Nevada, Las Vegas.

I also agree that the author may execute the standard contract with ProQuest for storage and reproduction of the completed thesis, including the materials to which I hold copyright

January 29, 2019

Signature	Date
Karl Kingsley, PhD, MPH	Professor
Name (typed)	Title

Appendix C

Permission to Use Copyrighted Material

University of Nevada, Las Vegas

Reserved for second manuscript

Curriculum Vitae

Graduate College

University of Nevada, Las Vegas

Jason Klingler, D.M.D.

Email: jasonklinglerdmd@gmail.com

Degrees:

Bachelor of Business Management, 2012
Brigham Young University

Doctor of Dental Medicine, 2016
University of Nevada, Las Vegas

Thesis Title:

Prevalence of Aggregatibacter Actinomycemcomitans and Fusobacterium Nucleatum Among Clinical Orthodontic Saliva Samples

Thesis Examination Committee:

Chairperson, Karl Kingsley, Ph.D. M.P.H.
Committee Member, Tanya Al-Talib, D.D.S., M.S.
Committee Member, Brian Chrzan, D.D.S., Ph.D.
Graduate Faculty Representative, Jennifer Pharr Ph.D.
Graduate Coordinator, Brian Chrzan, D.D.S., Ph.D.