May 2023

Oral Prevalence of Akkermancia Muciniphila Among Pediatric and Adult Orthodontic and Non-Orthodontic Patients

Ching Shen

ORAL PREVALENCE OF AKKERMANCIA MUCINIPHILA AMONG PEDIATRIC AND ADULT ORTHODONTIC AND NON-ORTHODONTIC PATIENTS

By

Ching Shen

Bachelor of Science – Microbiology, Immunology, and Molecular Genetics
University of California, Los Angeles
2014

Doctor of Dental Medicine
University of Nevada, Las Vegas
2020

A thesis submitted in partial fulfillment
of the requirements for the

Master of Science – Oral Biology

School of Dental Medicine
The Graduate College

University of Nevada, Las Vegas
May 2023

UNLV | GRADUATE COLLEGE

Thesis Approval

The Graduate College
The University of Nevada, Las Vegas

March 3, 2023

This thesis prepared by

Ching Shen

entitled

Oral Prevalence of Akkermancia Muciniphila Among Pediatric and Adult Orthodontic and Non-Orthodontic Patients

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

Alyssa Crittenden, Ph.D.
Vice Provost for Graduate Education & Dean of the Graduate College

Katherine Howard, Ph.D.
Examination Committee Member

Brian Chrzan, Ph.D.
Examination Committee Member

Maxim Gakh, J.D.
Graduate College Faculty Representative

Abstract

ORAL PREVALENCE OF AKKERMANCIA MUCINIPHILA AMONG PEDIATRIC AND ADULT ORTHODONTIC AND NON-ORTHODONTIC PATIENTS

By

Ching Shen

Dr. Karl Kingsley and Dr. Katherine Howard, Advisory Committee Chairs
Professor of Biomedical Sciences
Director of Student Research
University of Nevada, Las Vegas
School of Dental Medicine

Akkermansia muciniphila (AM) is one of many highly abundant intestinal microbes that influences homeostasis and metabolic disorders and may also play a role in oral disorders. However, there is little evidence regarding the oral prevalence of this organism. Based upon this lack of evidence, the primary goal of this project is to survey an existing saliva repository to determine the overall prevalence of this organism and any associations with demographic or patient characteristics (age, sex, body mass index, race/ethnicity, orthodontic therapy). Using an approved protocol, a total $n = 141$ pediatric samples from an existing saliva repository were screened using qPCR revealing 29.8% harbored AM with nearly equal distribution among males and females, $p = 0.8347$. Significantly higher percentages of pediatric, non-orthodontic patients were positive for AM (42.3%) compared with age-matched orthodontic patients (14.3%)-which

were equally distributed among non-orthodontic males (42.1%) and non-orthodontic females (42.5%). In addition, analysis of the adult samples revealed that nearly equal percentages of males (18.2%) and females (16.7%) harbored detectable levels of salivary AM, $p = 0.2035$. However, a higher proportion of non-orthodontic adult samples harbored AM (21.3%) compared to orthodontic samples (12.8%, $p = 0.0001$), which was equally distributed among males and females. These results suggest that both age and the presence of orthodontic brackets may influence microbial composition and, more specifically, are associated with reduction in AM among both pediatric and adult populations from their baseline levels.

Acknowledgments

I would like to extend my deepest gratitude to my committee chairs, Dr. Karl Kingsley and Dr. Katherine Howard, for their guidance and assistance with this project. I would also like to thank my committee members, Dr. Brian Chrzan and Dr. Maxim Gakh, for all of their help and support throughout this process.

Table of Contents

Abstract iii

Acknowledgments v

Table of Contents vi

List of Tables viii

List of Figures ix

Chapter 1: Introduction 1

Background and Significance 1

Research Question 2

Methods 2

Data Analysis 6

Timeline for Completion 8

References 9

Chapter 2 11

Abstract 11

Introduction 12

Materials and Methods 14

Results 18

Discussion 29

Conclusions 31

References 33

Chapter 3: Summary and Conclusions 41

Limitations and Recommendations 43

Appendix A 45

Appendix B 47

Curriculum Vitae 48

List of Tables

Chapter 1

Table 1. Study sample collection period..... 3

Chapter 2

Table 1. Demographic analysis of pediatric study samples..... 19

Table 2. Demographic analysis of adult study samples..... 20

Table 3. Analysis of DNA from study samples..... 21

Table 4. Analysis of AM-positive and AM-negative pediatric samples 23

Table 5. Analysis of AM-positive and AM-negative adult samples 25

List of Figures

Chapter 2

Figure 1. Heat map of qPCR pediatric study sample screening for *A. muciniphila* (AM) 22

Figure 2. Heat map of qPCR adult study sample screening for *A. muciniphila* (AM) 24

Figure 3. Age-associated difference in oral microbial prevalence 26

Figure 4. Orthodontic-associated differences in oral microbial prevalence 28

Chapter 1: Introduction

Background and Significance

Akkermansia muciniphila (*A. muciniphila*) is a newly discovered gram-negative anaerobe that is capable of degrading and using mucins as the sole source of nutrients (including carbon and nitrogen) [1]. Recent evidence has demonstrated that while oral and intestinal or "gut" concentrations of *A. muciniphila* may be correlated with being overweight or obese, other studies have demonstrated that *A. muciniphila* concentrations may also be correlated with metabolic control and improvement of glucose and lipid profiles in patients with Type II diabetes [2,3].

A. muciniphila has also recently been identified as an oral and intestinal commensal bacterium that can significantly reduce inflammation associated with periodontal pathogens, such as *Porphyromonas gingivalis* (*P. gingivalis*) [4]. Although orthodontic treatment has the potential to increase both *P. gingivalis* levels and periodontal inflammation in some patients, virtually nothing is known about the oral prevalence or levels of *A. muciniphila* among Orthodontic patients [5,6]. Based upon the lack of information regarding oral prevalence and distribution of this organism among Orthodontic patients – the overall goal of this project will be to screen an existing saliva repository for the presence (and levels) of *A. muciniphila*.

Research Question

Based upon this information, this project will strive to answer the following research question:

Will there be differences in the prevalence of *Akkermansia muciniphila* among Orthodontic patient saliva samples compared to non-Orthodontic samples?

H_0: There will be no difference in the prevalence of *Akkermansia muciniphila* between Orthodontic and non-Orthodontic patient saliva samples

H_A: There will be differences in the prevalence of *Akkermansia muciniphila* between Orthodontic and non-Orthodontic patient saliva samples

Methods

Institutional review board (IRB) approval will be sought to screen an existing saliva repository. Orthodontic and non-Orthodontic saliva samples will be identified for analysis. DNA will be isolated from these samples and tested for the presence of both human and bacterial DNA. Samples will subsequently be screened for *Akkermansia muciniphila* using organism-specific primers.

Current analysis of saliva repository

Several current and on-going Pediatric and Orthodontic resident projects are underway, which involve the screening of existing saliva samples for the presence of microbial pathogens. A review of data from previously approved IRB studies reveals a total of n = 1,176 saliva samples that were collected between 2011 and 2019 (Table 1).

Table 1. Study sample collection period

Collection period	Number of samples
2011 – 2012	*n* = 87
2012 – 2013	*n* = 152
2013 – 2014	*n* = 78
2014 – 2015	*n* = 133
2015 – 2016	*n* = 65
2016 – 2017	*n* = 136
2017 – 2018	*n* = 198
2018 – 2019	*n* = 229
2019 - 2020	*n* = 98
Total	*n* = 1176

A physical survey of the -80°C and -20°C freezers has identified available saliva samples with DNA already isolated *n* = 431, which were available for DNA quantity and DNA quality screening. Two other Orthodontic residents performed this screening, which has revealed only *n* = 63/431 or 14.6% of the available DNA isolates are of sufficient quality or quantity for analysis. This represents only 5.3% of the total samples available (*n* = 63/1176).

In order to generate a sufficient number of clinical samples for this study, DNA must be isolated from the remaining saliva samples in cold storage (-80°C, -20°C). This represents a maximum of *n* = 1,113 samples (*n* = 1176 – 63) that would require DNA isolation and screening for sufficient quality and quantity.

Each batch of DNA isolations must be performed using a refrigerated centrifuge - with the only available model in the lab currently holding 24 samples at a time. The estimated time for extraction, including centrifugation steps is approximately 2 -3 hours per set of 24 – which

means working both available research days per week (Tuesday / Friday) would require 4 – 6 weeks to yield n = 240 samples (less than one quarter of those available).

Power Analysis and Sample Size calculation

To determine the appropriate sample size for this type of PCR screening for microbial composition using DNA extracted from saliva, the recovery rate from the sample-limited step of DNA extraction can be used (90-95%) to establish the minimum expected difference of 0.10 or 10%. Using a significance level of $\alpha = 0.05$ and a power $p = 0.90$, a minimum sample size of fifty (n = 100) was calculated. Because this project involves the analysis and comparison of Orthodontic and non-Orthodontic samples, the overall number of samples needed would be approximately n = 100 (100 Orthodontic and 100 non-Orthodontic). Due to the estimates of the recovery rate (90% - 95%), it is currently estimated that approximately n = 220 samples would be needed for DNA isolation to provide the final estimated sample size of n = 200.

Analysis of DNA samples

Following DNA isolation, each sample must then be assessed for DNA quantity and DNA quality or purity. Quantitative or qPCR requires strict standards for both DNA purity (absorbance ratios at A260 nm and A280 nm >1.75). Any samples not meeting these stringent requirements will need to be processed again through the DNA isolation protocol to reduce contaminants and increase quality.

Although 5 – 50 ng is the suggested standard for DNA in any qPCR reaction, each sample must be screened in triplicate for at least two successive pathogens (*A. muciniphila, P. gingivalis*).

These data suggest 0.25 – 0.5 ug of DNA will be needed to ensure adequate quantity. Once again, any samples not meeting these requirements will need to be processed again through the DNA isolation protocol to increase quantity.

DNA quantity and quality can be assessed using the NanoDrop 2000 spectrophotometer. Samples containing 1-2 uL of DNA can be evaluated one-at-a-time on this instrument, which provides qualitative and quantitative data regarding each sample.

qPCR screening

Following the isolation of DNA from patient saliva samples, each of those samples meeting DNA quality and quantity standards will then be screened for the presence of *A. muciniphila* microbial DNA. There are currently no commercial kits (e.g. TaqMan assays) pre-packaged with primers specific for this microbe, so each qPCR reaction will need to be done individually.

Screening of approximately 200 samples in triplicate, n = 600 (200 x 3) will require significant manual input time to complete for the *A. muciniphila* screening. Similarly, each sample will then also need to be screened separately for the presence of *P. gingivalis*. This will require an additional 600 individual qPCR reactions.

In addition, microbial standards (16s rRNA) will be required for each individual reaction, which will result in the addition of approximately 1,200 qPCR reactions. This will be needed to establish a positive control and provide reference standards to allow for the semi-quantitative analysis of *A. muciniphila* and *P. gingivalis* DNA in each sample.

Data Analysis

PCR data can be quantified to determine the relative starting concentrations of the target sequence. This can provide a semi-quantitative or approximate measure of the bacterial levels from each sample. This method has been used to provide correlations (Pearson's correlation or R) between PCR band intensity and starting levels of bacterial DNA. Comparisons of bacterial levels between Orthodontic and non-Orthodontic patients can be measured using Students t-tests, which are appropriate for measured data such as PCR band intensity.

Descriptive statistics and preliminary analysis

Analysis of these data will include several successive layers or iterations. First, and most importantly – these data will be sorted by qPCR screening results. Identification of the *A. muciniphila*-positive and -negative samples and the matching and correlation with *P. gingivalis*-positive and negative results will provide initial descriptive statistics about prevalence of these organisms.

Analysis of these data will also provide some information regarding the coexistence of these bacteria among both Orthodontic and non-Orthodontic patient samples. In addition, the levels of each bacteria can be evaluated to determine if overall levels or percentage of overall bacteria are different among the positive and negative samples, as well as among those testing single or double positive. These data can be further sorted to provide more detailed information regarding additional independent (predictor) variables.

Additional predictor variables

An analysis of these independent (predictor) variables will also provide more information regarding the prevalence of these microbes within these patient samples. For example, the basic information of clinical status (Orthodontic versus non-Orthodontic) may provide some information regarding the potential role of brackets in any differences observed between these populations. Moreover, there are many other variables that might significantly impact these analyses.

Another variable is age of the patient. Comparison of adult patients versus pediatric patients may provide some additional information regarding the distribution and prevalence of these organisms and the relationship with growth and development. Analysis of specific ages (younger teenagers versus older teenagers) may provide significantly more information regarding the influence of age (if any) and these organisms and their interactions within the oral cavity.

Another example is sex of the patient. Comparison of male and female patients within the Orthodontic and non-Orthodontic populations can provide yet more information regarding the potential role of hormonal influences on the prevalence of these bacteria. Further analysis by age and gender and microbial presence or microbial level will provide more granular details regarding the influence of sex and hormones on microbial presence or levels.

One further example is race and ethnicity. Many studies have demonstrated the role of race and ethnicity in the US, which may be tied to socioeconomic status in many regions of the country. An analysis and comparison of age, sex and race/ethnicity combined with the analysis of

Orthodontic versus non-Orthodontic and microbial prevalence would provide an unprecedented level of detail regarding the potential factors that might disproportionately affect microbial levels among these patients.

Timeline for completion

Each stage of this project will require a significant investment of time and resources. The first stage, identification of samples within the repository containing sufficient saliva for DNA isolation may take a few weeks to accomplish. The second relates step of DNA isolation may take a few months (4 – 6 weeks minimum possibly extending up to 12 weeks depending upon lab availability and COVID restrictions).

Sample screening for DNA purity and quantity will be the next significant step, which will require significant time investment. Each sample must be screened and loaded separately and individually by hand. Previous experience with this protocol has yielded an estimated timeline of approximately 2 – 3 weeks. Any samples not meeting purity or quantity standards will need to undergo additional DNA isolation and rescreening – thereby extending the overall time needed to complete this step.

qPCR screening for both microbial pathogens in triplicate, along with the DNA microbial standards (16s rRNA) will require approximately 2,400 reactions, which must be set up by hand given the lack of prepackaged and prepared TaqMan assays for these microbes. 200 x 3 = 600 for both pathogens (1,200) and matching 16s rRNA standards for comparison.

Data analysis may be the most time consuming portion of this project, given the number of independent (predictor) variables for evaluation. Clinical status (Orthodontic versus non-Orthodontic), Age, Sex and Race/Ethnicity will need to be separately evaluated and then analyzed in various combinations to determine, which (if any) are associated with microbial prevalence or concentration of either or both pathogens. This may take several months to accomplish.

References

1. Cheng D, Xie M. A review of a potential and promising probiotic candidate - Akkermansia muciniphila. J Appl Microbiol. 2020 Oct 28. doi: 10.1111/jam.14911. Epub ahead of print. PMID: 33113228.
2. Crovesy L, Masterson D, Rosado EL. Profile of the gut microbiota of adults with obesity: a systematic review. Eur J Clin Nutr. 2020 Sep;74(9):1251-1262. doi: 10.1038/s41430-020-0607-6. Epub 2020 Mar 30. PMID: 32231226.
3. Cao TTB, Wu KC, Hsu JL, Chang CS, Chou C, Lin CY, Liao YM, Lin PC, Yang LY, Lin HW. Effects of Non-insulin Anti-hyperglycemic Agents on Gut Microbiota: A Systematic Review on Human and Animal Studies. Front Endocrinol (Lausanne). 2020 Sep 23;11:573891. doi: 10.3389/fendo.2020.573891. PMID: 33071980; PMCID: PMC7538596.
4. Huck O, Mulhall H, Rubin G, Kizelnik Z, Iyer R, Perpich JD, Haque N, Cani PD, de Vos WM, Amar S. Akkermansia muciniphila reduces Porphyromonas gingivalis-induced inflammation and periodontal bone destruction. J Clin Periodontol. 2020 Feb;47(2):202-212. doi: 10.1111/jcpe.13214. Epub 2019 Dec 2. PMID: 31674689.

5. Calniceanu H, Stratul SI, Rusu D, Jianu A, Boariu M, Nica L, Ogodescu A, Sima L, Bolintineanu S, Anghel A, Milicescu S, Didilescu A, Roman A, Surlin P, Solomon S, Tudor M, Rauten AM. Changes in clinical and microbiological parameters of the periodontium during initial stages of orthodontic movement in patients with treated severe periodontitis: A longitudinal site-level analysis. Exp Ther Med. 2020 Dec;20(6):199. doi: 10.3892/etm.2020.9329. Epub 2020 Oct 14. PMID: 33123229; PMCID: PMC7588781.
6. Marothiya S, Jain U, Bharti C, Polke P, Agrawal P, Shah R, Mishra P. Evaluation of Changes in Microbiology and Periodontal Parameters During and After Fixed Orthodontic Appliances. Mymensingh Med J. 2020 Oct;29(4):983-990. PMID: 33116106.

Chapter 2

Oral Prevalence of *Akkermansia muciniphila* Differs among Pediatric and Adult Orthodontic and Non-Orthodontic Patients

This chapter has been submitted and accepted to *Microorganisms* and is presented in the style of that Journal. The complete Citation is:

Shen C, Clawson JB, Simpson J, Kingsley K. Oral Prevalence of *Akkermansia muciniphila* Differs among Pediatric and Adult Orthodontic and Non-Orthodontic Patients. *Microorganisms*. 2023 Jan 1;11(1):112. doi: 10.3390/microorganisms11010112. PMID: 36677404.

Role of Authors:

C.S., J.B.C. and J.S. were responsible for methodology, data curation, investigation, formal analysis, writing—original draft preparation. K.K. was responsible for conceptualization, methodology, resources, data curation, formal analysis, and supervision, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Abstract

Akkermansia muciniphila (AM) is one of many highly abundant intestinal microbes that influences homeostasis and metabolic disorders and may also play a role in oral disorders. However, there is little evidence regarding the oral prevalence of this organism. Based upon this lack of evidence, the primary goal of this project is to survey an existing saliva repository to determine the overall prevalence of this organism and any associations with demographic or patient characteristics (age, sex, body mass index, race/ethnicity, orthodontic therapy). Using an approved protocol, a total $n = 141$ pediatric samples from an existing saliva repository were screened using qPCR revealing 29.8% harbored AM with nearly equal distribution among males and females, $p = 0.8347$. Significantly higher percentages of pediatric, non-orthodontic patients were positive for AM (42.3%) compared with age-matched orthodontic patients (14.3%)—which were equally distributed among non-orthodontic males (42.1%) and non-orthodontic females (42.5%). In addition, analysis of the adult samples revealed that nearly equal percentages of

males (18.2%) and females (16.7%) harbored detectable levels of salivary AM, $p = 0.2035$. However, a higher proportion of non-orthodontic adult samples harbored AM (21.3%) compared to orthodontic samples (12.8%, $p = 0.0001$), which was equally distributed among males and females. These results suggest that both age and the presence of orthodontic brackets may influence microbial composition and, more specifically, are associated with reduction in AM among both pediatric and adult populations from their baseline levels.

Keywords: *Akkermansia muciniphila*; saliva screening; orthodontic; oral prevalence

Introduction

Akkermansia muciniphila is one of many highly abundant human intestinal microbes that may have the ability to significantly influence homeostasis and metabolic disorders [1,2]. For example, some evidence has demonstrated that decreased abundance of the mucin-degrading *Akkermansia* allows for dysbiosis among other microbial competitors that negatively affect host metabolic syndrome and immune system responses [3,4]. Systematic reviews of this evidence have confirmed that reductions in *Akkermansia* are highly associated with metabolic syndrome as the inflammation-reducing properties of this microbe are lost, associations that may also be related to other disorders including obesity, hyperlipidemia and hypertension [5,6].

The functional ability of this microorganism to modulate inflammation, basal metabolism and other metabolic functions has led to many studies evaluating the potential for its use as a therapeutic treatment option [7,8]. Growing evidence has demonstrated the benefits and impacts of *Akkermansia* used as a probiotic to reduce inflammation and restore microbial homeostasis

and equilibrium in clinical settings [9–11]. However, recent studies have demonstrated that changes to the oral microbiota, most notably with *Akkermansia*, not only alter oral health but also precede and subsequently contribute to the development or prevention of these metabolic disorders [12,13].

More specifically, the presence of *Akkermansia* appears to down-regulate the production of inflammatory cytokines such as IL-10 and IL-12, as well as other inflammatory biomarkers associated with *Porphyromonas gingivalis*-associated periodontitis [14,15]. Additionally, other studies have now revealed that administration of *Akkermansia* may reduce both periodontal and systemic inflammation in a dose-dependent manner [16,17]. However, the only studies of prevalence to date have focused on evaluation of microbial composition or changes within the gut and intestinal tracts without any corresponding studies of oral microbial prevalence [18–20].

Given the ability of these microorganisms to influence oral as well as systemic disorders, it is surprising how little evidence is available regarding the prevalence and oral ecology of this organism [21]. Studies from this group have created a saliva biorepository that has been successfully used to conduct molecular screenings for other novel oral pathogens, including the cariogenic bacterium *Scardovia wiggsiae* and periodontal pathogen *Selenomonas noxia* [22–24]. Based upon this information, the primary goal of this project was to survey this existing saliva biorepository to determine the overall prevalence of *Akkermansia muciniphila* and any associations with demographic or patient characteristics, such as age, sex, race or ethnicity, and orthodontic treatment status.

Materials and Methods

Human Subjects Approval

This was a retrospective study involving the use of an existing biorepository. The protocol for this study was submitted, reviewed and approved by the Institutional Review Board (IRB) and the Office for the Protection of Research Subjects (OPRS) at the University of Nevada, Las Vegas (UNLV) as Research Exempt under Protocol #1717625-1 "Retrospective analysis of microbial prevalence from DNA isolated from saliva samples originally obtained from the University of Nevada, Las Vegas (UNLV) School of Dental Medicine (SDM) pediatric and clinical population" on 3 March 2021.

Original Collection Protocol

The study protocol used for the original collection of clinical saliva samples was reviewed and approved under protocol OPRS#1305-4466M "The Prevalence of Oral Microbes in Saliva from the UNLV School of Dental Medicine Pediatric and Adult Clinical Population" in 2013. In brief, adult and pediatric patients who agreed to participate provided Informed Consent and Pediatric Assent, if applicable. Up to 5.0 mL was collected in sterile polypropylene tubes, which were labeled with non-duplicated, randomly generated numbers to avoid the collection of any personal or patient-specific information.

Inclusion criteria were patients of record at UNLV-SDM who agreed to participate and provide Informed Consent and/or Pediatric Assent. Exclusion criteria included any person not a patient of record at UNLV-SDM and any patient who declined to participate. Samples were stored at −80

°C in a secured biomedical laboratory freezer. Basic demographic information was concurrently collected including patient age, sex, race or ethnicity, as well as orthodontic treatment status.

DNA Isolation and Analysis

DNA was isolated from all clinical samples using the phenol:chloroform extraction method using TRIzol DNA isolation reagent from ThermoFisher Scientific (Fair Lawn, NJ, USA), as previously described [22–24]. Briefly, samples were thawed and 400 uL of saliva was removed and placed into a sterile microcentrifuge tube with an equal volume of TRIzol reagent and triturated before adding 200 uL of chloroform and incubated for ten minutes on ice. Samples were then centrifuged at 12,000× *g* or relative centrifugal force (RCF) for 15 min using an Eppendorf Refrigerated Microcentrifuge obtained from Fisher Scientific (Fair Lawn, NJ, USA). The upper aqueous phase was transferred to a sterile microcentrifuge tube with an equal volume of isopropanol to precipitate the DNA and mixed thoroughly. Samples were centrifuged for 10 min and isopropanol was removed. Samples were washed with molecular grade Ethanol and centrifuged for five minutes. Ethanol was removed and samples were resuspended using nuclease-free distilled water.

DNA was screened using the NanoDrop 2000 Spectrophotometer from Fisher Scientific (Fair Lawn, NJ, USA). Absorbance readings at A260 nm and A280 nm were used to determine the purity and concentration of DNA. Samples with sufficient quantity (>10 ng) and quality as determined by the A260:A280 ratio (>1.65) were selected for qPCR screening.

qPCR Screening

Screening of samples was performed using the QuantStudio Real-Time Polymerase Chain Reaction (PCR) system from Applied Biosciences (Waltham, MA, USA). Screening qPCR reactions utilized SYBR Green qPCR Master Mix from ThermoFisher Scientific (Fair Lawn, NJ, USA), which consisted of ABsolute SYBR Green (12.5 uL), nuclease-free water (7.5 uL), forward and reverse primers (1.75 uL each), and sample DNA (1.5 uL) diluted to 1.0 ng/uL for a total reaction volume of 25 uL. Cycle specifications included activation of the enzyme at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C (15 s), annealing using each primer pair-specific temperature (30 s), with final extension at 72 °C (30 s). Validated primer sets included [22–29]:

Positive control, bacterial 16S rRNA

Forward 16S rRNA primer: 5′-ACG CGT CGA CAG AGT TTG ATC CTG GCT-3′

27 nt, 56% GC content, Tm = 76 °C

Reverse 16S rRNA primer: 5′-GGG ACT ACC AGG GTA TCT AAT-3′

21 nt, 48% GC content, Tm = 62 °C

Akkermansia muciniphila (AM)

Forward AM primer: 5′-CAG CAC GTG AAG GTG GGG-3′

18 nt, 67% GC content, Tm = 69 °C

Reverse AM primer: 5′-CCT TGG GGT TGG CTT CAG AT-3′

20 nt, 55% GC content, Tm = 68 °C

Aggregatibacter actinomycetemcomitans (AA)

Forward AA primer, 5′-ATT GGG GTT TAG CCC TGG T-3′

19 nt, 53% GC, Tm = 67 °C

Reverse AA primer, 5′-GGC ACA AAC CCA TCT CTG A-3′

19 nt, 53%GC, Tm = 65 °C

Fusobacterium nucleatum (FN)

Forward FN primer; 5′-CGC AGA AGG TGA AAG TCC TGT AT-3′

23 nt, 48% GC, Tm = 67 °C

Reverse FN primer; 5′-TGG TCC TCA CTG ATT CAC ACA GA-3′

23 nt, 48% GC, Tm = 68 °C

Selenomonas noxia (SN)

Forward SN primer: 5′-TCT GGG CTA CAC ACG TAC TAC AAT G-3′

25 nt, 48% GC, Tm = 68 °C

Reverse SN primer: 5′-GCC TGC AAT CCG AAC TGA GA-3′

20 nt, 55% GC, Tm = 68 °C

Porphyromonas gingivalis (PG)

Forward PG primer: 5′-TAC CCA TCG TCG CCT TGG T = 3′

19 nt, 58% GC, Tm = 69 °C

Reverse PG primer: 5′-CGG ACT AAA ACC GCA TAC ACT TG-3′

23 nt, 48% GC, Tm = 66 °C

Statistical Analysis

Descriptive statistics for demographic variables, including number and percentage of males and females, average age with range, and race or ethnicity were compiled in Microsoft Excel (Redmond, WA, USA). Samples were screened and further categorized as AM-positive and AM-negative for analysis using Chi Square statistics, which is appropriate for non-parametric (categorical data) analysis. A significance level of alpha = 0.05 was used for all calculations.

Results

A total of n = 227 clinical saliva samples were identified for inclusion in this study. Analysis of the samples from the pediatric population (n = 141) revealed approximately half were derived from females (51.1%), which closely matched the overall clinic demographics (52.8%), p = 0.6891 (Table 1). Evaluation of the racial and ethnic demographic data revealed the majority of samples were derived from minority (non-White) patients (70.2%), which also approximates the overall pediatric clinic population (75.3%), p = 0.2482. The average age of the pediatric study sample was 13.14 years, which was significantly higher than the overall clinical population age of 10.44 years mainly due to the original sampling protocol which restricted sample collection to patients age seven and older, p = 0.021.

Table 1. Demographic analysis of pediatric study samples.

Demographic	Pediatric Study Sample (*n*=141)	Clinic Population	Statistical Analysis
Sex			
Pediatric–Female	(*n*=72/141) 51.1%	52.8%	χ^2=0.160, d.f.=1
Pediatric–Male	(*n*=69/141) 48.9%	47.2%	*p*=0.6891
Race/Ethnicity			
Pediatric - White	(*n*=42/141) 29.8%	24.7%	χ^2=1.333,d.f.=1
Pediatric - Minority	(*n*=99/141) 70.2%	75.3%	*p*=0.2482
Pediatric - Hispanic	(*n*=74/141) 52.5%	52.1%	
Age			
Average (Median)	13.14 yrs. (12 yrs.)	10.44 yrs. (10 years)	Two-tailed *t*-test *p*=0.021
Range	7 - 17 yrs.	0 - 17 yrs.	

Analysis of the samples from the adult population (*n* = 86) revealed approximately half were derived from females (48.8%), which closely matched the overall clinic demographics (49.1%), *p* = 0.9883 (Table 2). Evaluation of the racial and ethnic demographic data revealed the majority of samples were derived from minority (non-White) patients (62.8%), which also approximates the overall adult clinic population (65.4%), *p* = 0.0893. Finally, the average age of the adult study sample was 46.35 years, which was significantly higher than the overall clinical population age of 42.31 years, *p* = 0.028.

Table 2. Demographic analysis of adult study samples.

Demographic	Adult Study Sample (*n*=86)	Clinic Population	Statistical Analysis
Sex			
Adult–Female	(*n*=42/86) 48.8%	49.1%	χ^2=0.016, d.f.=1
Adult–Male	(*n*=44/86) 51.2%	50.9%	*p*=0.9883
Race/Ethnicity			
Adult–White	(*n*=32/86) 37.2%	34.6%	χ^2=2.987, d.f.=1
Adult–Minority	(*n*=54/86) 62.8%	65.4%	*p*=0.0839
Adult–Hispanic	(*n*=47/86) 54.7%	58.6%	
Age			
Average (Median)	46.35 yrs. (42 yrs.)	42.31 yrs. (41 yrs.)	Two-tailed *t*-test *p*=0.028
Range	18 - 73 yrs.	18 - 89 yrs.	

The DNA isolated from the pediatric and adult saliva samples was screened using spectrophotometric analysis to determine the suitability of each sample for qPCR screening (Table 3). These data revealed that the average DNA concentration from the pediatric saliva samples was sufficient for qPCR screening (average 481.2 ng/uL), which was within the range specified by the manufacturer for isolation from biological samples (100–1000 ng/uL). In addition, the quality of samples was also suitable for qPCR screening (A260:A280 ratio = 1.73 average.) Similarly, the concentration (434.1 ng/uL) and quality (A260:A280 ratio = 1.75 average) of DNA from adult saliva samples was also sufficient for qPCR screening and analysis.

Table 3. Analysis of DNA from study samples.

Study sample	DNA concentration	DNA purity (A260:A280 ratio)
Pediatric samples *n*=141	481.2 ng/uL +/- 55.1 127.2 - 769.1 ng/uL	Average: 1.73 Range: 1.69 - 1.82
Adult samples *n*=86	434.1 ng/uL +/- 72.2 169.3 - 872.8 ng/uL	Average: 1.75 Range: 1.71 - 1.84

Molecular screening of pediatric study samples revealed *n* = 42/141 or 29.8% harbored DNA specific for *Akkermansia* or AM (Figure 1). Approximately one-third exhibited cycle threshold detection levels in the high (C16–C20, *n* = 3/42 or 7.1%), moderate-high C21–C25, *n* = 7/42 or 16.7%) or moderate (C26–C30, *n* = 4/42 or 9.5%) range. In contrast, two-thirds (*n* = 28/42 or 66.7%) exhibited cycle threshold detection levels in the low-moderate (C31–C35, *n* = 15/42 or 35.7%) or low (C36–C40, *n* = 13/42 or 31.0%) range.

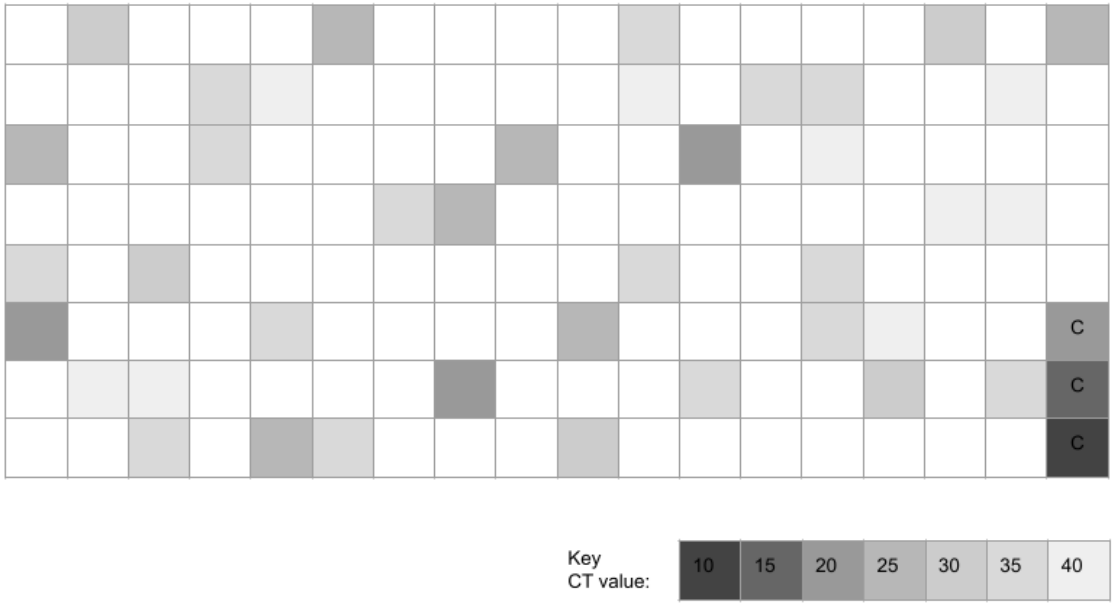

Figure 1. Heat map of qPCR pediatric study sample screening for *Akkermansia muciniphila* (AM). This screening revealed *n* = 42/141 or 29.8% of samples harbored DNA specific for AM. The majority of these samples (*n* = 28/42 or 66.7%) exhibited AM-specific DNA levels corresponding to low (C36–C40) or moderately low (C31–C35) cycle threshold detection values. C = positive control standard curve data for 16S rRNA.

More detailed analysis of these pediatric samples revealed that nearly equal percentages of males (*n* = 21/69 or 30.4%) and females (*n* = 21/72 or 29.2%) harbored detectable levels of salivary AM, *p* = 0.8347 (Table 4). Interestingly, sorting by orthodontic status revealed that a higher proportion of non-orthodontic samples harbored AM (*n* = 33/78 or 42.3%) than orthodontic samples (*n* = 9/63 or 14.3%). More specifically, more males (*n* = 16/38 or 42.1%) and females (*n* = 17/40 or 42.5%) from non-orthodontic samples harbored AM than age-matched orthodontic samples from males (*n* = 5/31 or 16.1%) or females (*n* = 4/32 or 12.5%), *p* = 0.0001.

Table 4. Analysis of AM-positive and AM-negative pediatric samples.

Demographic	AM-positive	AM-negative	Statistical analysis
Pediatric–Males	*n*=21/69 or 30.4%	*n*=48/69 or 69.6%	χ^2=0.044, d.f.=1
Pediatric–Females	*n*=21/72 or 29.2%	*n*=51/72 or 70.8%	*p*=0.8347
Total	*n*=42/141 or 29.8%	*n*=99/141 or 70.2%	
Pediatric–Males Non-Orthodontic	*n*=16/38 or 42.1%	*n*=22/38 or 57.9%	χ^2=758.853, d.f.=1
Pediatric–Males Orthodontic	*n*=5/31 or 16.1%	*n*=26/31 or 83.9%	*p*=0.0001
Total	*n*=21/69 or 30.4%	*n*=48/69 or 69.6%	
Pediatric–Females Non-Orthodontic	*n*=17/40 or 42.5%	*n*=23/40 or 57.5%	χ^2=822.857, d.f.=1
Pediatric–Females Orthodontic	*n*=4/32 or 12.5%	*n*=28/32 or 87.5%	*p*=0.0001
Total	*n*=21/72 or 29.2%	*n*=51/72 or 70.8%	
Non-Orthodontic	*n*=33/78 or 42.3%	*n*=45/78 or 57.6%	χ^2=639.734, d.f.=1
Orthodontic	*n*=9/63 or 14.3%	*n*=54/63 or 85.7%	*p*=0.0001
Total	*n*=42/141 or 29.8%	*n*=99/141 or 70.2%	

Molecular screening of adult study samples revealed *n* = 15/86 or 17.4% harbored DNA specific for AM (Figure 2). Only four samples exhibited cycle threshold detection levels in the high (C16–C20, *n* = 1/15 or 6.7%), moderate-high C21–C25, *n* = 0/15 or 0.0%) or moderate (C26–C30, *n* = 3/15 or 20.0%) range. In contrast, nearly three-fourths (*n* = 11/15 or 73.3%) exhibited cycle threshold detection levels in the low-moderate (C31–C35, *n* = 5/15 or 33.3%) or low (C36–C40, *n* = 6/15 or 40.0%) range.

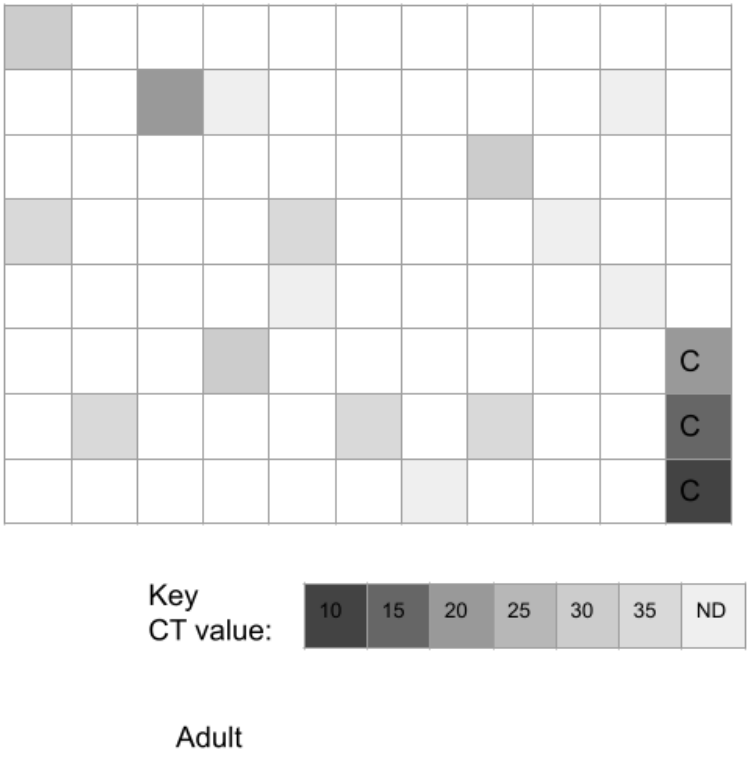

Figure 2. Heat map of qPCR adult study sample screening for *Akkermansia muciniphila* (AM). This screening revealed *n* = 15/86 or 17.4% of samples harbored DNA specific for AM. The majority of these samples (*n* = 11/15 or 73.3%) exhibited AM-specific DNA levels corresponding to low (C36–C40) or moderately low (C31–C35) cycle threshold detection values. C = positive control standard curve data for 16S rRNA.

More detailed analysis of the adult samples revealed that nearly equal percentages of males (*n* = 8/44 or 18.2%) and females (*n* = 7/42 or 16.7%) harbored detectable levels of salivary AM, *p* = 0.2035 (Table 5). Analysis of these data using orthodontic status revealed that a higher proportion of non-orthodontic, adult samples harbored AM (*n* = 10/47 or 21.3%) than orthodontic samples (*n* = 5/39 or 12.8%). More specifically, more adult males (*n* = 5/24 or

20.8%) and females (*n* = 5/23 or 21.7%) from non-orthodontic samples harbored AM than age-matched orthodontic samples from males (*n* = 3/20 or 15%) or females (*n* = 2/19 or 10.5%), *p* = 0.0001.

Table 5. Analysis of AM-positive and AM-negative adult samples.

Demographic	AM-positive	AM-negative	Statistical analysis
Adult–Males	*n*=8/44 or 18.2%	*n*=36/44 or 81.8%	χ^2=1.617, d.f.=1
Adult–Females	*n*=7/42 or 16.7%	*n*=35/42 or 83.3%	*p*=0.2035
Total	*n*=15/86 or 17.4%	*n*=71/86 or 82.6%	
Adult–Males Non-Orthodontic	*n*=5/24 or 20.8%	*n*=19/24 or 79.2%	χ^2=26.384, d.f.=1
Adult–Males Orthodontic	*n*=3/20 or 15.0%	*n*=17/20 or 85.0%	*p*=0.0001
Total	*n*=8/44 or 18.2%	*n*=36/44 or 81.8%	
Adult–Females Non-Orthodontic	*n*=5/23 or 21.7%	*n*=18/23 or 78.3%	χ^2=133.482, d.f.=1
Pediatric–Females Orthodontic	*n*=2/19 or 10.5%	*n*=17/19 or 89.5%	*p*=0.0001
Total	*n*=7/42 or 16.7%	*n*=35/42 or 83.3%	
Non-Orthodontic	*n*=10/47 or 21.3%	*n*=37/47 or 78.7%	χ^2=64.731, d.f.=1
Orthodontic	*n*=5/39 or 12.8%	*n*=34/39 or 87.2%	*p*=0.0001
Total	*n*=15/86 or 17.4%	*n*=71/86 or 82.6%	

To determine if the changes in oral prevalence were affected by age, differences between the pediatric and adult samples were analyzed (Figure 3). These data demonstrated that a large difference in the oral prevalence of AM was observed between the pediatric, non-orthodontic

(42.3%) and adult, non-orthodontic (21.3%) samples at −21%. Further analysis of prevalence among pediatric orthodontic (14.3%) and adult orthodontic (12.8%) revealed a slight difference of only −1.5%.

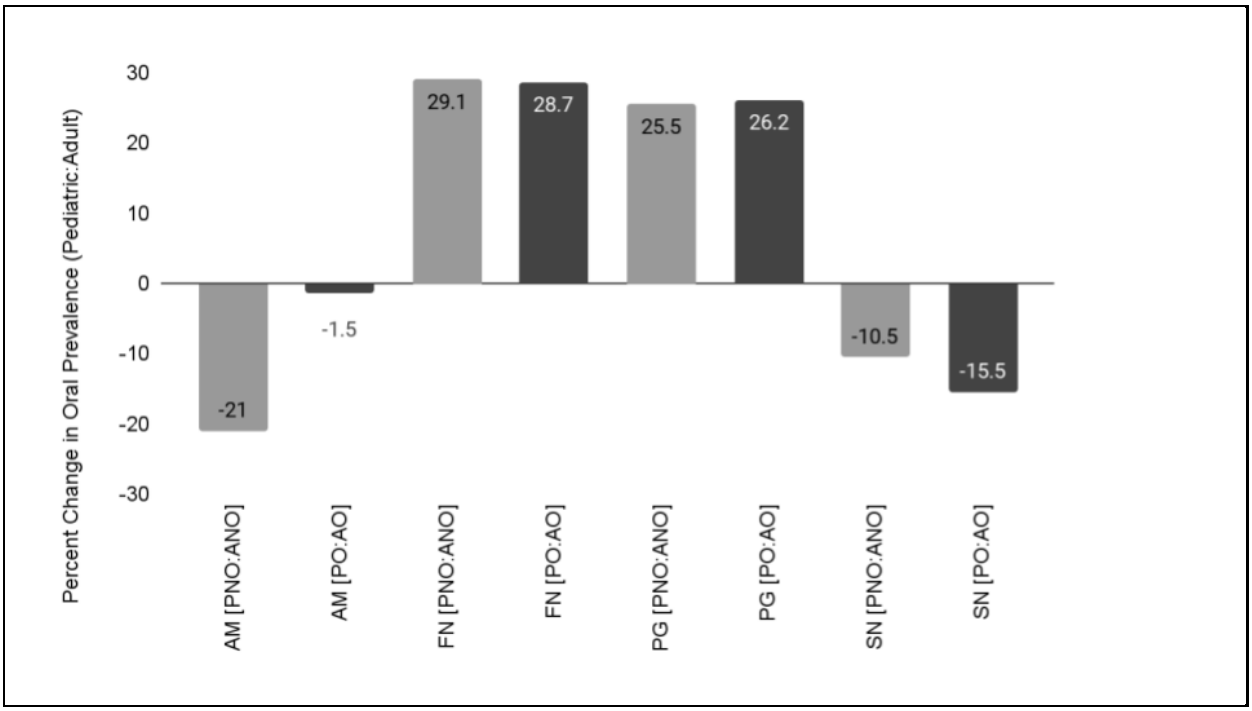

Figure 3. Age-associated difference in oral microbial prevalence. Decreased oral prevalence between pediatric and adult samples was observed for *Akkermansia muciniphila* (AM) with the largest difference observed between the non-orthodontic samples. In addition, other age-related changes were observed with *Fusobacterium nucleatum* (FN), *Porphyromonas gingivalis* (PG) and *Selenomonas noxia* (SN), although those differences were more similar in direction (positive or negative) and magnitude between orthodontic and non-orthodontic samples.

To determine if these age-associated effects were restricted to this oral microbial population, previously collected data from other studies of Gram-negative oral microbes *Fusobacterium nucleatum* (FN), *Porphyromonas gingivalis* (PG), and *Selenomonas noxia* (SN) were also plotted

and graphed. These data also demonstrated differences between the pediatric and adult samples, such as the differences in FN between the pediatric, non-orthodontic (33%) and adult, non-orthodontic (62.1%) samples at 29.1% that closely matched the differences between pediatric, orthodontic (38.1%) and adult, orthodontic (66.8%) samples at 28.7%. In addition, differences were observed in the oral prevalence of PG between the pediatric, non-orthodontic (28.9%) and adult, non-orthodontic (54.4%) samples by 25.5% that also closely matched the differences between pediatric, orthodontic (45.2%) and adult, orthodontic (71.4%) samples at 26.2%. Finally, age-associated differences were observed with the oral prevalence of SN between pediatric, non-orthodontic (16.6%) and adult, non-orthodontic (5.5%) samples at −10.5, while differences between pediatric, orthodontic (28%) and adult, orthodontic (12.5%) samples were approximately −15.5%.

To determine if the changes in oral prevalence were correlated with orthodontic therapy, differences between the orthodontic and non-orthodontic samples were analyzed (Figure 4). These data demonstrated that a large difference in the oral prevalence of AM was observed between the pediatric non-orthodontic (42.3%) and pediatric orthodontic (14.3%) samples of −28.0%. In addition, the difference in oral prevalence between adult, non-orthodontic (21.3%) and adult, orthodontic (12.8%) samples was found to be −8.5%.

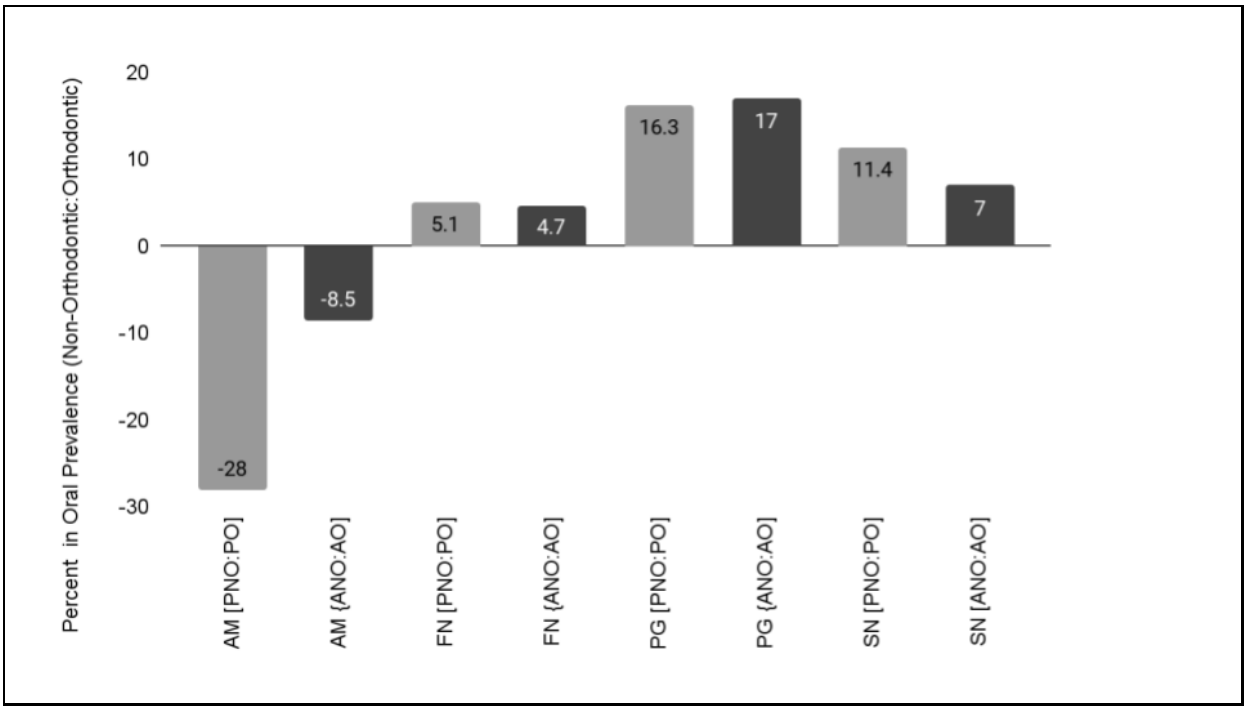

Figure 4. Orthodontic-associated differences in oral microbial prevalence. Decreased oral prevalence between non-orthodontic and orthodontic samples was observed for *Akkermansia muciniphila* (AM) with the largest differences observed between the pediatric samples. In addition, other orthodontic-related changes were observed with *Fusobacterium nucleatum* (FN), *Porphyromonas gingivalis* (PG) and *Selenomonas noxia* (SN), although those differences were more similar in direction (positive or negative) and magnitude between adult and pediatric samples.

To determine if these orthodontic-associated differences were restricted to this oral microbial population, data from other studies of Gram-negative FN, PG, and SN were also plotted and graphed. These data also demonstrated differences between the orthodontic and non-orthodontic samples, such as the differences in FN between the pediatric, non-orthodontic (33%) and pediatric, orthodontic (38.1%) samples at 5.1% and between the adult, non-orthodontic (62.1%) and adult, orthodontic (66.8%) samples at 4.7%. In addition, differences were observed in the

oral prevalence of PG between the pediatric, non-orthodontic (28.9%) and pediatric, orthodontic (45.2%) samples by 16.3%, as well as the adult, non-orthodontic (54.4%) and adult, orthodontic (71.4%) samples at 17%. Finally, orthodontic-associated differences were also observed with the oral prevalence of SN between pediatric, non-orthodontic (16.6%) and pediatric, orthodontic (28%) samples at 11.4%, while differences between adult, non-orthodontic (5.5%) and adult, orthodontic (12.5%) samples were approximately 7%.

Discussion

The principal objective of this study was to evaluate the oral prevalence of *Akkermansia* and to uncover any correlations with patient demographics, such as age, sex, or orthodontic treatment status. This study successfully evaluated more than 225 clinical saliva samples, making it one of the largest and most comprehensive oral prevalence surveys ever undertaken at this institution [24,30]. In addition, due to the lack of evidence regarding oral prevalence of this organism, any studies that provide insight into the factors that may influence the distribution within populations could help us to understand how and when this microbial constituent colonizes the gastrointestinal tract—further modulating the microbiome and health of the host [30,31].

The findings of this study demonstrating that *Akkermansia* appears to be most prevalent among pediatric samples may be particularly relevant given that recent evidence suggesting that microbial diversity including *Akkermansia* is strongly associated with metabolic health in children, particularly among those who are overweight and obese [32,33]. In addition, these results combined with the results of the only other study to date evaluating microbial prevalence of *Akkermansia* among orthodontic patients, greatly increases the overall number of patients evaluated with and without orthodontic appliances [34]. Research has demonstrated that

orthodontic therapy shifts oral microbial composition particularly among overweight and obese, adolescent patients [35]. These results may provide some of the first observations of these shifts among this specific patient population. In fact, recent studies from this group have found that body mass index (BMI) has been steadily increasing among the pediatric and adolescent patient population, with most recent average BMI ranging between 25.6 (overweight) and 31.3 (obese) [36,37].

Additional studies from this group have evaluated the role of orthodontic brackets in altering microbial populations, including *Selenomonas noxia*, *Scardovia wiggsiae*, *Streptococcus mutans* and *Porphyomonas gingivalis* [23–25,38,39]. The comparative analysis undertaken in this current study demonstrates that age-related changes in the oral prevalence of Gram-negative *Akkermansia* are similar to that of Gram-negative *Selenomonas* (higher levels among children than adults) but may exhibit opposite change with the presence of orthodontic appliances (*Akkermansia* decreased, *Selenomonas* increased), confirmed by other reports from this group reporting *Selenomonas* prevalence among orthodontic and non-orthodontic patients [26,27]. Furthermore, the finding that *Akkermansia* prevalence decreased among orthodontic patients while the periodontal pathogens *Fusobacterium* and *Porphyromonas* increased, may suggest that the mechanisms that drive these changes may be separate and distinct from changes in the periodontium and gingival crevices normally observed in orthodontic therapy [28,29].

Despite the significance of these findings, there are several limitations of this study which should also be considered. First, this is a retrospective study of clinical samples from an existing biorepository that may have some pre-existing differences in demographics due to the sample

collection protocols of those initial study sample collection protocols [22–24]. In addition, most of the clinical patient population at this publicly funded university-based dental school are low-income and minority patients that may also have additional challenges and barriers to access and care that may have influenced the outcomes of this study—although no clinical measures such as periodontal pocket depth (PPD), plaque index (PI) or decayed-missing-filled teeth (DMFT) score were available among these retrospective samples for analysis [25,38,39]. Some evidence from this institution also suggests that oral microbial prevalence may be strongly shifting over time, which was not a primary outcome variable analyzed for in the current study and may have influenced these findings [40]. Due to the limited scale of this project and limited financial support for this preliminary study, other methodologies such as next generation sequencing were not employed—although this was unlikely to affect the outcome of this study. Finally, research has demonstrated that medically compromised patients may also suffer from changes in microbial load and may be an important patient population for future studies of this nature [41].

Conclusions

This study provides strong evidence that *Akkermansia* prevalence shifts with age, with younger patients more likely to harbor detectable levels in saliva that could potentially seed the gastrointestinal tract and influence gut microbial composition later in life. In addition, this study found that presence of orthodontics dramatically shifted *Akkermansia* prevalence among both pediatric and adult populations, which did not correlate with shifts of other known periodontal pathogens. This provides some of the first evidence that orthodontic therapy may be associated with changes in oral *Akkermansia* prevalence but may be related to the shifts in other oral microbial communities rather than the known changes in gingivitis and periodontitis typically associated with orthodontic treatment.

Author Contributions

C.S., J.B.C. and J.S. were responsible for methodology, data curation, investigation, formal analysis, writing—original draft preparation. K.K. was responsible for conceptualization, methodology, resources, data curation, formal analysis, supervision, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding. The APC was funded by the Office of Research at the University of Nevada, Las Vegas—School of Dental Medicine and the Department of Advanced Education—Orthodontic Dental Residency Program. Karl Kingsley is co-investigator on the National Institute of Health (NIH) grant R15DE028431.

Institutional Review Board Statement

This study was conducted according to the guidelines of the Declaration of Helsinki. and was reviewed and approved by the University of Nevada, Las Vegas (UNLV) Institutional review board (IRB) under protocol 1619329-1 titled “Retrospective analysis of Oral Health Status of Dental Population” on 24 July 2020. This retrospective analysis of previously collected data regarding the UNLV School of Dental Medicine (SDM) pediatric patient population was deemed Exempt pursuant to the Basic Health and Human Services (HHS) Policy for the Protection of Human Research Subjects (46.101) regarding IRB exemption for research that involves the study of existing data, documents or records that current exist and are not prospectively collected and 1. Participants cannot be directly identified; and 2. Participants cannot be identified through identifiers linked to them.

Informed Consent Statement

Informed Consent was waived due to the exemption to retrospective human subjects research (46.101) regarding IRB exemption for research involving existing samples in which subjects cannot be identified directly or through identifiers.

Data Availability Statement

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the study protocol data protection parameters requested by the IRB and OPRS for the initial study approval.

Acknowledgments

The authors would like to acknowledge the presentation of preliminary data from this manuscript by J.B.C. and J.S. at the International Association for Dental Research (IADR) conference in 2021.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Karamzin AM, Ropot AV, Sergeyev OV, Khalturina EO. Akkermansia muciniphila and host interaction within the intestinal tract. Anaerobe. 2021 Dec;72:102472. doi: 10.1016/j.anaerobe.2021.102472. Epub 2021 Oct 29. PMID: 34743983.
2. Geerlings SY, Kostopoulos I, de Vos WM, Belzer C. Akkermansia muciniphila in the Human Gastrointestinal Tract: When, Where, and How? Microorganisms. 2018 Jul

23;6(3):75. doi: 10.3390/microorganisms6030075. PMID: 30041463; PMCID: PMC6163243.

3. Aggarwal V, Sunder S, Verma SR. Disease-associated dysbiosis and potential therapeutic role of Akkermansia muciniphila, a mucus degrading bacteria of gut microbiome. Folia Microbiol (Praha). 2022 May 20:1–14. doi: 10.1007/s12223-022-00973-6. Epub ahead of print. PMID: 35596115; PMCID: PMC9122250.
4. de Vos WM, Tilg H, Van Hul M, Cani PD. Gut microbiome and health: mechanistic insights. Gut. 2022 May;71(5):1020-1032. doi: 10.1136/gutjnl-2021-326789. Epub 2022 Feb 1. PMID: 35105664; PMCID: PMC8995832.
5. Michels N, Zouiouich S, Vanderbauwhede B, Vanacker J, Indave Ruiz BI, Huybrechts I. Human microbiome and metabolic health: An overview of systematic reviews. Obes Rev. 2022 Apr;23(4):e13409. doi: 10.1111/obr.13409. Epub 2022 Jan 3. PMID: 34978141.
6. Vallianou N, Christodoulatos GS, Karampela I, Tsilingiris D, Magkos F, Stratigou T, Kounatidis D, Dalamaga M. Understanding the Role of the Gut Microbiome and Microbial Metabolites in Non-Alcoholic Fatty Liver Disease: Current Evidence and Perspectives. Biomolecules. 2021 Dec 31;12(1):56. doi: 10.3390/biom12010056. PMID: 35053205; PMCID: PMC8774162.
7. Xu Y, Wang N, Tan HY, Li S, Zhang C, Feng Y. Function of Akkermansia muciniphila in Obesity: Interactions With Lipid Metabolism, Immune Response and Gut Systems. Front Microbiol. 2020 Feb 21;11:219. doi: 10.3389/fmicb.2020.00219. PMID: 32153527; PMCID: PMC7046546.
8. Macchione IG, Lopetuso LR, Ianiro G, Napoli M, Gibiino G, Rizzatti G, Petito V, Gasbarrini A, Scaldaferri F. Akkermansia muciniphila: key player in metabolic and

gastrointestinal disorders. Eur Rev Med Pharmacol Sci. 2019 Sep;23(18):8075-8083. doi: 10.26355/eurrev_201909_19024. PMID: 31599433.

9. Cheng HL, Yen GC, Huang SC, Chen SC, Hsu CL. The next generation beneficial actions of novel probiotics as potential therapeutic targets and prediction tool for metabolic diseases. J Food Drug Anal. 2022 Mar 15;30(1):1-10. doi: 10.38212/2224-6614.3396. PMID: 35647717.
10. Dudík B, Kiňová Sepová H, Greifová G, Bilka F, Bílková A. Next generation probiotics: an overview of the most promising candidates. Epidemiol Mikrobiol Imunol. 2022 Spring;71(1):48-56. English. PMID: 35477270.
11. Shen H, Zhao Z, Zhao Z, Chen Y, Zhang L. Native and Engineered Probiotics: Promising Agents against Related Systemic and Intestinal Diseases. Int J Mol Sci. 2022 Jan 6;23(2):594. doi: 10.3390/ijms23020594. PMID: 35054790; PMCID: PMC8775704.
12. Minty M, Canceil T, Serino M, Burcelin R, Tercé F, Blasco-Baque V. Oral microbiota-induced periodontitis: a new risk factor of metabolic diseases. Rev Endocr Metab Disord. 2019 Dec;20(4):449-459. doi: 10.1007/s11154-019-09526-8. PMID: 31741266.
13. Graves DT, Corrêa JD, Silva TA. The Oral Microbiota Is Modified by Systemic Diseases. J Dent Res. 2019 Feb;98(2):148-156. doi: 10.1177/0022034518805739. Epub 2018 Oct 25. PMID: 30359170; PMCID: PMC6761737.
14. Huck O, Mulhall H, Rubin G, Kizelnik Z, Iyer R, Perpich JD, Haque N, Cani PD, de Vos WM, Amar S. Akkermansia muciniphila reduces Porphyromonas gingivalis-induced inflammation and periodontal bone destruction. J Clin Periodontol. 2020 Feb;47(2):202-212. doi: 10.1111/jcpe.13214. Epub 2019 Dec 2. PMID: 31674689.

15. Tavares CO, Rost FL, Silva RBM, Dagnino AP, Adami B, Schirmer H, de Figueiredo JAP, Souto AA, Maito FDM, Campos MM. Cross Talk between Apical Periodontitis and Metabolic Disorders: Experimental Evidence on the Role of Intestinal Adipokines and Akkermansia muciniphila. J Endod. 2019 Feb;45(2):174-180. doi: 10.1016/j.joen.2018.10.013. PMID: 30711174.
16. Mulhall H, DiChiara JM, Deragon M, Iyer R, Huck O, Amar S. Akkermansia muciniphila and Its Pili-Like Protein Amuc_1100 Modulate Macrophage Polarization in Experimental Periodontitis. Infect Immun. 2020 Dec 15;89(1):e00500-20. doi: 10.1128/IAI.00500-20. PMID: 33020212; PMCID: PMC7927938.
17. Mulhall H, DiChiara JM, Huck O, Amar S. Pasteurized Akkermansia muciniphila reduces periodontal and systemic inflammation induced by Porphyromonas gingivalis in lean and obese mice. J Clin Periodontol. 2022 Jul;49(7):717-729. doi: 10.1111/jcpe.13629. Epub 2022 May 3. PMID: 35415929.
18. Tarallo S, Ferrero G, De Filippis F, Francavilla A, Pasolli E, Panero V, Cordero F, Segata N, Grioni S, Pensa RG, Pardini B, Ercolini D, Naccarati A. Stool microRNA profiles reflect different dietary and gut microbiome patterns in healthy individuals. Gut. 2022 Jul;71(7):1302-1314. doi: 10.1136/gutjnl-2021-325168. Epub 2021 Jul 27. PMID: 34315772; PMCID: PMC9185830.
19. Becken B, Davey L, Middleton DR, Mueller KD, Sharma A, Holmes ZC, Dallow E, Remick B, Barton GM, David LA, McCann JR, Armstrong SC, Malkus P, Valdivia RH. Genotypic and Phenotypic Diversity among Human Isolates of Akkermansia muciniphila. mBio. 2021 May 18;12(3):e00478-21. doi: 10.1128/mBio.00478-21. PMID: 34006653; PMCID: PMC8262928.

20. Yan J, Sheng L, Li H. Akkermansia muciniphila: is it the Holy Grail for ameliorating metabolic diseases? Gut Microbes. 2021 Jan-Dec;13(1):1984104. doi: 10.1080/19490976.2021.1984104. PMID: 34674606; PMCID: PMC8726741.
21. Singh H, Torralba MG, Moncera KJ, DiLello L, Petrini J, Nelson KE, Pieper R. Gastro-intestinal and oral microbiome signatures associated with healthy aging. Geroscience. 2019 Dec;41(6):907-921. doi: 10.1007/s11357-019-00098-8. Epub 2019 Oct 16. PMID: 31620923; PMCID: PMC6925087.
22. Emett J, David R, McDaniel J, McDaniel S, Kingsley K. Comparison of DNA Extracted from Pediatric Saliva, Gingival Crevicular Fluid and Site-Specific Biofilm Samples. Methods Protoc. 2020 Jul 9;3(3):48. doi: 10.3390/mps3030048. PMID: 32660039; PMCID: PMC7565886.
23. McDaniel J, McDaniel S, Samiano BJ, Marrujo M, Kingsley K, Howard KM. Microbial Screening Reveals Oral Site-Specific Locations of the Periodontal Pathogen Selenomonas noxia. Curr Issues Mol Biol. 2021 Jun 12;43(1):353-364. doi: 10.3390/cimb43010029. PMID: 34204609; PMCID: PMC8929098.
24. McDaniel S, McDaniel J, Howard KM, Kingsley K. Molecular Screening and Analysis Reveal Novel Oral Site-Specific Locations for the Cariogenic Pathogen Scardovia wiggsiae. Dent J (Basel). 2021 Jun 17;9(6):73. doi: 10.3390/dj9060073. PMID: 34204219; PMCID: PMC8234915.
25. 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; PMCID: PMC3473244.

26. Dhillon N, Kingsley K, Howard KM. Prevalence of Selenomonas noxia among Pediatric and Adult Orthodontic Patients. International Journal of Research and Reports in Dentistry. 2019, 2(1): 1-7.
27. Kim N, Trumbo M, Perkins P, Foote K, Samiano BJ, Marrujo M, Kingsley K, Howard KM. Comparing the Incidence and Prevalence of Oral Microbial Pathogens Selenomonas noxia and Streptococcus mitis within the UNLV-SDM Clinical Patient Population. International Journal of Research and Reports in Dentistry. 4(2): 1-12, 2021.
28. 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, 2019, 2:9. doi: 10.28933/ijdrr-2019-01-2405.
29. Klingler J, Prevalence of Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum among Clinical Orthodontic and Non-Orthodontic Saliva Samples. Doctoral Dissertation, University of Nevada, Las Vegas, Paradise, NV, USA, 2019. doi: 10.34917/15778485
30. de Vos WM. Microbe Profile: Akkermansia muciniphila: a conserved intestinal symbiont that acts as the gatekeeper of our mucosa. Microbiology (Reading). 2017 May;163(5):646-648. doi: 10.1099/mic.0.000444. Epub 2017 May 22. PMID: 28530168.
31. Luo Y, Lan C, Li H, Ouyang Q, Kong F, Wu A, Ren Z, Tian G, Cai J, Yu B, He J, Wright AG. Rational consideration of Akkermansia muciniphila targeting intestinal

health: advantages and challenges. NPJ Biofilms Microbiomes. 2022 Oct 17;8(1):81. doi: 10.1038/s41522-022-00338-4. PMID: 36253412; PMCID: PMC9576740.

32. Alcazar M, Escribano J, Ferré N, Closa-Monasterolo R, Selma-Royo M, Feliu A, Castillejo G, Luque V; Obemat2.0 Study Group. Gut microbiota is associated with metabolic health in children with obesity. Clin Nutr. 2022 Aug;41(8):1680-1688. doi: 10.1016/j.clnu.2022.06.007. Epub 2022 Jun 15. PMID: 35777107.
33. Sugino KY, Ma T, Paneth N, Comstock SS. Effect of Environmental Exposures on the Gut Microbiota from Early Infancy to Two Years of Age. Microorganisms. 2021 Oct 13;9(10):2140. doi: 10.3390/microorganisms9102140. PMID: 34683461; PMCID: PMC8537618.
34. Wirth R, Maróti G, Lipták L, Mester M, Al Ayoubi A, Pap B, Madléna M, Minárovits J, Kovács KL. Microbiomes in supragingival biofilms and saliva of adolescents with gingivitis and gingival health. Oral Dis. 2022 Oct;28(7):2000-2014. doi: 10.1111/odi.13883. Epub 2021 May 6. PMID: 33876475.
35. Sharara SH, Cleaver LM, Saloom H, Carpenter GH, Cobourne MT. Salivary bacterial community profile in normal-weight and obese adolescent patients prior to orthodontic treatment with fixed appliances. Orthod Craniofac Res. 2022 Nov;25(4):569-575. doi: 10.1111/ocr.12571. Epub 2022 May 1. PMID: 35298866.
36. Su T, Jackson M, Sacry K, Kingsley K. Longitudinal Analysis of Body Mass Index (BMI) Trends among a Pediatric Dental Patient Population. EC Paediatrics 11.5 (2022): 20-28.
37. Frayna C, Devantier C, Harris B, Kingsley K, Polanski JM. Education Regarding and Adherence to Recommended Nutrition Guidelines among Dental Students. Dent J

(Basel). 2021 Aug 9;9(8):93. doi: 10.3390/dj9080093. PMID: 34436005; PMCID: PMC8393618.

38. Milne W, Rezaei G, Whiteley A, Kingsley K. Cariogenic pathogen Scardovia wiggsiae screening among pediatric orthodontic patients: A pilot Study. Curr. Res. Dent. 2018;9:1-5. doi: 10.3844/crdsp.2018.1.5
39. Reyes N, Pollock A, Whiteley A, Kingsley K, Howard KM. Prevalence of Scardovia wiggsiae among a Pediatric Orthodontic Patient Population. EC Dent. Sci. 2017;2:203-10.
40. Kornhaber MS, Florence T, Davis T, Kingsley K. Assessment of Oral Human Papillomavirus Prevalence in Pediatric and Adult Patients within a Multi-Ethnic Clinic Population. Dent J (Basel). 2022 Apr 1;10(4):54. doi: 10.3390/dj10040054. PMID: 35448048; PMCID: PMC9031267.
41. Pawlaczyk-Kamieńska T, Borysewicz-Lewicka M, Batura-Gabryel H. Salivary Biomarkers and Oral Microbial Load in Relation to the Dental Status of Adults with Cystic Fibrosis. Microorganisms. 2019 Dec 13;7(12):692. doi: 10.3390/microorganisms7120692. PMID: 31847106; PMCID: PMC6955745.

Chapter 3: Summary and Conclusions

The principal objective of this study was to evaluate the oral prevalence of *Akkermansia* and to uncover any correlations with patient demographics, such as age, sex, or orthodontic treatment status. This study successfully evaluated more than 225 clinical saliva samples, making it one of the largest and most comprehensive oral prevalence surveys ever undertaken at this institution [24,30]. In addition, due to the lack of evidence regarding oral prevalence of this organism, any studies that provide insight into the factors that may influence the distribution within populations could help us to understand how and when this microbial constituent colonizes the gastrointestinal tract—further modulating the microbiome and health of the host [30,31].

The findings of this study demonstrating that *Akkermansia* appears to be most prevalent among pediatric samples may be particularly relevant given that recent evidence suggesting that microbial diversity including *Akkermansia* is strongly associated with metabolic health in children, particularly among those who are overweight and obese [32,33]. In addition, these results combined with the results of the only other study to date evaluating microbial prevalence of *Akkermansia* among orthodontic patients, greatly increases the overall number of patients evaluated with and without orthodontic appliances [34]. Research has demonstrated that orthodontic therapy shifts oral microbial composition particularly among overweight and obese, adolescent patients [35]. These results may provide some of the first observations of these shifts among this specific patient population. In fact, recent studies from this group have found that body mass index (BMI) has been steadily increasing among the pediatric and adolescent patient population, with most recent average BMI ranging between 25.6 (overweight) and 31.3 (obese) [36,37].

Additional studies from this group have evaluated the role of orthodontic brackets in altering microbial populations, including *Selenomonas noxia*, *Scardovia wiggsiae*, *Streptococcus mutans* and *Porphyomonas gingivalis* [23–25,38,39]. The comparative analysis undertaken in this current study demonstrates that age-related changes in the oral prevalence of Gram-negative *Akkermansia* are similar to that of Gram-negative *Selenomonas* (higher levels among children than adults) but may exhibit opposite change with the presence of orthodontic appliances (*Akkermansia* decreased, *Selenomonas* increased), confirmed by other reports from this group reporting *Selenomonas* prevalence among orthodontic and non-orthodontic patients [26,27]. Furthermore, the finding that *Akkermansia* prevalence decreased among orthodontic patients while the periodontal pathogens *Fusobacterium* and *Porphyromonas* increased, may suggest that the mechanisms that drive these changes may be separate and distinct from changes in the periodontium and gingival crevices normally observed in orthodontic therapy [28,29].

Overall, this study provides strong evidence that *Akkermansia* prevalence shifts with age, with younger patients more likely to harbor detectable levels in saliva that could potentially seed the gastrointestinal tract and influence gut microbial composition later in life. In addition, this study found that presence of orthodontics dramatically shifted *Akkermansia* prevalence among both pediatric and adult populations, which did not correlate with shifts of other known periodontal pathogens. This provides some of the first evidence that orthodontic therapy may be associated with changes in oral *Akkermansia* prevalence but may be related to the shifts in other oral microbial communities rather than the known changes in gingivitis and periodontitis typically associated with orthodontic treatment.

Based on the findings presented in this study, the null hypothesis can be rejected and the alternative hypothesis can be accepted for the following research question:

Will there be differences in the prevalence of *Akkermansia muciniphila* among Orthodontic patient saliva samples compared to non-Orthodontic samples?

H_0: There will be no difference in the prevalence of *Akkermansia muciniphila* between Orthodontic and non-Orthodontic patient saliva samples

H_A: There will be differences in the prevalence of *Akkermansia muciniphila* between Orthodontic and non-Orthodontic patient saliva samples

Limitations and Recommendations

As this is a retrospective study of clinical samples from an existing biorepository, there may be pre-existing differences in demographics due to the sample collection protocols of those initial study sample collection protocols. Clinical measures such as periodontal pocket depth (PPD), plaque index (PI) or decayed-missing-filled teeth (DMFT) score are also not available. In addition, most of the clinical patient population at this publicly funded university-based dental school are low-income and minority patients that may also have additional challenges and barriers to access and care that may have influenced the outcomes of this study.

Some evidence from this institution also suggests that oral microbial prevalence may be strongly shifting over time, which was not a primary outcome variable analyzed for in the current study and may have influenced these findings. Due to the limited scale of this project and limited financial support for this preliminary study, other methodologies such as next generation

sequencing were not employed. Finally, research has demonstrated that medically compromised patients may also suffer from changes in microbial load and may be an important patient population for future studies of this nature.

Appendix A

UNLV

UNLV Biomedical IRB - Administrative Review
Notice of Excluded Activity

DATE:	March 3, 2021
TO:	Karl Kingsley, PhD, MPH
FROM:	UNLV Biomedical IRB
PROTOCOL TITLE:	[1717625-1] Retrospective analysis of microbial prevalence from DNA isolated from saliva samples originally obtained from the University of Nevada, Las Vegas (UNLV) School of Dental Medicine (SDM) pediatric and clinical population
SUBMISSION TYPE:	New Project
ACTION:	EXCLUDED - NOT HUMAN SUBJECTS RESEARCH
REVIEW DATE:	March 3, 2021
REVIEW TYPE:	Administrative Review

Thank you for your submission of New Project materials for this protocol. This memorandum is notification that the protocol referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The UNLV Biomedical IRB has determined this protocol does not meet the definition of human subjects research under the purview of the IRB according to federal regulations. It is not in need of further review or approval by the IRB.

We will retain a copy of this correspondence with our records.

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

If you have questions, please contact the Office of Research Integrity - Human Subjects at IRB@unlv.edu or call 702-895-2794. Please include your protocol title and IRBNet ID in all correspondence.

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

UNLV

UNLV Biomedical IRB - Administrative Review
Notice of Excluded Activity

DATE:	July 24, 2020
TO:	Karl Kingsley
FROM:	UNLV Biomedical IRB
PROTOCOL TITLE:	[1619329-1] Retrospective analysis of Oral Health Status of Dental Population
SUBMISSION TYPE:	New Project
ACTION:	EXCLUDED - NOT HUMAN SUBJECTS RESEARCH
REVIEW DATE:	July 24, 2020
REVIEW TYPE:	Administrative Review

Thank you for your submission of New Project materials for this protocol. This memorandum is notification that the protocol referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The UNLV Biomedical IRB has determined this protocol does not meet the definition of human subjects research under the purview of the IRB according to federal regulations. It is not in need of further review or approval by the IRB.

We will retain a copy of this correspondence with our records.

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

If you have questions, please contact the Office of Research Integrity - Human Subjects at IRB@unlv.edu or call 702-895-2794. Please include your protocol title and IRBNet ID in all correspondence.

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 **Oral Prevalence of *Akkermansia muciniphila* Differs among Pediatric and Adult Orthodontic and Non-Orthodontic Patients**, authored by **Ching Shen, James Brigham Clawson, Justin Simpson,** and **Karl Kingsley** originally published in ***Microorganisms,* January 2023**, 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

Ching Shen	February 21, 2023
Signature	Date
Ching Shen	Resident
Name (typed)	Title

Curriculum Vitae

Graduate College

University of Nevada, Las Vegas

Ching Shen

Email: jshen8d@gmail.com

Degrees:
Bachelor of Science – Microbiology, Immunology, and Molecular Genetics
Asian Languages Minor
University of California, Los Angeles
2014

Doctor of Dental Medicine
University of Nevada, Las Vegas
2020

Thesis Title:
Oral Prevalence of *Akkermansia muciniphila* among Pediatric and Adult Orthodontic and Non-Orthodontic Patients

Thesis Examination Committee:
Chairperson, Karl Kingsley, Ph.D. M.P.H.
Chairperson, Katherine Howard, Ph.D.
Committee Member, Brian Chrzan, D.D.S., Ph.D.
Graduate Faculty Representative, Maxim Gakh, J.D., M.P.H.
Graduate Coordinator, Brian Chrzan, D.D.S., Ph.D.