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CHARACTERIZING MICROBIOME CHANGES IN THE GRADUATE STUDENT GUT

A THESIS APPROVED FOR THE
DEPARTMENT OF ANTHROPOLOGY

BY

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Dedicated to Joan-Rivers, Beyoncé, and Meredith.



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Table of Contents

Acknowledgements ..... **v**

List of Tables ..... **vii**

List of Figures ..... **viii**

Abstract ..... **ix**

Background **1**

The golden age of microbiome science 2
The gut microbiome 5
The oral microbiome 6

Materials and Methods **8**

Participants, Sample and Data Collection 8
Samples 10
16S Sequencing and Data Processing 11
Comparative Data 12

Results **17**

Graduate Students' Microbiome analysis 17
Population comparison analysis 19

Discussion **32**

References **37**

Appendix I: Supplementary Tables **42**

Appendix II: Supporting Documents **46**

List of Tables

Table 1 – Graduate Student Microbiome (GSG) Participant Data 14

Table 2 – Non-Native Norman, OK Population 15

Table 3 – Cheyenne & Arapaho, OK Population 16

Table S1 – Graduate Student Microbiome (GSG) participant survey data 42

Table S2 – Graduate Student Microbiome (GSG) biometric data.....43

Table S3 – Norman, OK (NOR) control population metadata.....44

Table S4 – Cheyenne & Arapaho, OK (C&A) population metadata.....45

List of Figures

Figure 1 – Phylum level changes in graduate students' microbiomes 23

Figure 2 – Changes in graduate students' gut microbe *Ruminococcaceae* between summer and winter 24

Figure 3 – Alpha-diversity comparisons 25

Figure 4 – Graduate student beta-diversity by geography 26

Figure 5 – Beta-diversity comparisons of gut microbiomes 27

Figure 6 – Beta-diversity comparison across populations by age.....28

Figure 7 – Boxplots of relative abundance of oral taxa (genus level).....29

Figure 8 – Boxplots of relative abundance of gut taxa (genus level) 30

Figure 9 – Boxplots of relative abundance of taxa (family level) 31

Abstract

Dysbiosis of the human microbiome is linked to(Turnbaugh et al. 2006) human health problems, and as such, is a main concern of anthropological microbiome research. Analysis of how microbiomes change over time and under stress may reveal trends that lead to dysbiotic states. For this particular study, graduate students are of interest because they often relocate to distant places to study in their field of expertise. For any human, we can expect that travel and a new regional diet may influence the microbiome. For new graduate students, the added stress of school could also have a considerable influence. The purpose of this study is to determine if the combined effects of diet, travel, and stress are detectable in the oral and gut microbiomes of first year graduate students at the University of Oklahoma. Eleven participants, males and females, between the ages of 18-25 self-collected fecal and saliva samples and were surveyed about life style behaviors. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) and deep sequenced using Next-Generation sequencing (NGS) to characterize the taxonomic profiles of the gut and oral microbiomes. Though the results were not statistically significant, the study participants show an increase over time in alpha-diversity of the gut microbiome and only minimal change in the oral microbiome. The 16S rRNA sequence data show that the microbiomes of graduate students did experience change during their first semester at school, but the pattern of change is complex and generally not

consistent across individuals. Most significantly, *Ruminococcaceae* is enriched in the winter samples. This study continues to characterize the adaptive nature of the human microbiome; future work would benefit from a larger participant cohort.

Background

The term *microbiome* has been coined to describe the ubiquitous nature of microbial communities and their ecological niches (Lederberg and Mccray 2001). Human microbiomes have changed over the course of human evolution (Warinner et al. 2015; Zilber-Rosenberg and Rosenberg 2008), and they are also influenced by everyday behaviors and lifestyle choices (Jeon et al. 2013). Human microbiomes are important because they have been associated in a variety of health issues including, obesity (Turnbaugh et al. 2006), rheumatoid arthritis (Scher and Abramson 2011), asthma (Chen and Blaser 2008), irritable bowel syndrome (Tana et al. 2010), and periodontitis (Costalonga and Herzberg 2014). Diet plays a key role in shaping the microbiome (Moeller et al. 2014); both the microbiome and diet can be influenced by travel (Dey et al. 2015) and stress (Dash et al. 2015; Moloney et al. 2014). Travel, stress, and dietary changes are often majors factors of change for graduate students; as such, the microbiomes of graduate students are of interest in that they may exhibit these stressors as microbial community changes. It is common for graduate students to participate in microbiome studies, but a study focusing specifically on graduate students has not yet been performed, to my knowledge.

The purpose of this study is to characterize change in community structure of the oral and gut microbiomes of first-year graduate students at the University of Oklahoma to understand the possible effects of stress, travel, and diet in a university setting. Graduate students often relocate to distant

places to study in their field. Travel and a new regional diet may influence the microbiome. For new graduate students the added stress of school and relocation can considerably influence dietary choices, and as a result, may impact the microbiome. For these reasons, graduate students are an ideal population to analyze the severity of impact of relocation and chronic stress on microbiomes. I hypothesize that the stressors associated with the impact of graduate student life will be evident as changes in the oral and gut microbiomes of the students.

The golden age of microbiome science

Microbial science has progressed from a time when the goal of the researcher was to simply understand the relationship between a single pathogenic microbe and the human host. Nineteenth century researchers such as Louis Pasteur and Robert Koch, confirmed notions of the time that invisible organisms could be harmful to health. This work was paramount to evaluating human health in the context of infectious disease and it continues to inform health today. Culture dependent methods for identifying microbes were the gold standard during this era. Culturing microbes requires that the researcher isolate the single microbe of interest in pure culture and conduct an analysis based on the physiological or biochemical characteristics of the organism; however, this method becomes a limitation when the required conditions for growth of a particular microbe is poorly understood (Hiergeist et al. 2015). While much has been learned from culturing microorganisms, the extent of

non-pathogenic microbial interactions within the human body has often been overlooked.

Microbial research has been rapidly advancing with the introduction of culture independent techniques that have led to deeper taxonomic inventories for ecological characterization, making human microbiome research possible. In contrast to the culture dependent techniques, culture independent techniques directly examine molecular sequences. Analyzing DNA sequences using this method saves the researcher the effort of culturing each microbe contained within the sample and allows for the microbial community to be profiled. The 16S ribosomal RNA (rRNA) gene serves as an ideal genetic marker for microbial community analysis. This gene is shared by bacteria and archaea and has both very conserved regions, for targeting, and hyper-variable regions, for species characterization (Scholz et al. 2012; Woese et al. 1990). Targeted amplicon sequencing allows researchers to characterize entire microbial communities by directly analyzing and characterizing DNA extracted from a particular site or sample (Hiergeist et al. 2015).

Advancements in sequencing technology have also contributed to the scientific ability to analyze the complexity of biological material. Researchers pioneered the field of genetics through the use of the Sanger method of sequencing that provided a tool towards visualizing the sequence of nucleotides that make up DNA (Sanger and Coulson 1975). The Sanger method still provides longer reads at a higher quality than the newer methods (DiGuistini et al. 2009). The limitation of Sanger sequencing can be in its

low-throughput and limited of ability to provide sequences for samples of mixed communities.

Amassing a larger quantity of sequence data can be achieved through NGS techniques that provide researchers the ability to simultaneously sequence the DNA of mixed communities in one batch of chemistry. Microbiome science today finds itself at the intersection of NGS and 16S rRNA gene metataxonomic analysis. Samples of mixed communities can be barcoded, combined, and sequenced together as a pool to be later demultiplexed using identifying sequence barcodes (Caporaso et al. 2012). The combination of the high-throughput of NGS and the targeting and species identifying qualities of 16S rRNA gene sequences produce a snapshot of the microbial community contained within a sample. These advancements in methods and technology have ushered microbiome research to the forefront of science.

According to some estimates, human bodies are composed of only 10% human nucleated cells and 90% bacterial cells (Bianconi et al. 2013; Sender et al. 2016). The human body is, therefore, composed of more than one organism. The functions of these microbial organisms are in an early stage of scientific understanding, but what is known shows that these microbes are influential and essential to the lives of humans at every stage of life (Aagaard et al. 2014; Chen and Blaser 2008; Dominguez-Bello et al. 2010; Gilbert 2014; Gilbert et al. 2012; Park et al. 2015). Consequently, to fully understand human evolution, health and disease, the human biological self must be

studied as a collection of cells from different, yet often well integrated, organisms (Zilber-Rosenberg and Rosenberg 2008).

The gut microbiome

The human gut microbiome is a site of particular interest for its influence on health. Of the human body sites, the gut harbors the majority of our microbes (Sekirov et al. 2010). One example of an important role the gut microbiome plays is to assist in metabolic functions so that the host can process foods that would be otherwise difficult to digest (Karasov et al. 2011). In humans, some of our diet is broken down by enzymes and other digestive chemicals produced in the stomach and small intestine. Fibrous food can escape digestion while in transit to the large intestine; it is here that human gut microbes assist in digesting fiber by fermentation that produces short-chain fatty acids that the large intestine can absorb as nutrients (Stearns et al. 2011; Walter and Ley 2011). Researchers are learning that as diets have changed in humans and other animals over evolutionary time scales, so has the bacterial composition of their gut microbiome (Ley et al. 2008).

The human gut microbiome is an ecology that is influenced by many factors, and microbiomes adapt to various conditions, both internal and external. The majority of the human gut microbiome is made up of microbial members from two phyla: *Firmicutes* and *Bacteroidetes* (Mariat et al. 2009). This ecology is influenced by dietary composition as well as weight loss and weight gain (David et al. 2014). Seasonal changes can affect the availability of

food and the types of microbes present in the environment (Bowers et al. 2011; Davenport et al. 2014). The built environment also has an impact on the microbial composition, such as where we live and our living arrangements, and cohabitating couples, families, and dog owners share a certain number of personal microbes (Jeon et al. 2013; Lax et al. 2014; Song et al. 2013). Of particular interest to health sciences are factors that cause dysbiosis of the microbiome, which is a microbial imbalance that occurs when the symbiotic relationship of the host and microbes are disrupted, resulting in a diminished health status.

The oral microbiome

Whereas the gut microbiome contains the highest numerical abundance of microbes, the human oral microbiome contains a higher richness, or diversity, of microbes (Dewhirst et al. 2010; Stearns et al. 2011). Microbes in the oral cavity are plentiful because they are both transient, entering our bodies by way of food and the environment, as well as, endogenous, or native to the oral cavity. The oral and gut microbiomes are inter-related in that the oral cavity is the beginning of the digestive tract and gateway into the human gut. The oral cavity houses distinct microbial habitats (e.g. saliva, teeth, gingiva, tongue, cheek, lip, tonsil, pharynx, and esophagus) that vary in microbial community structure (Dewhirst et al. 2010; Lazarevic et al. 2012).

Oral microbes have been associated with a variety of health states (Dewhirst et al. 2010; He and Shi 2009; Jenkinson and Lamont 2005; Lazarevic et al. 2012). Two of the primary oral health issues that have been explored are dental caries and periodontal disease. The understanding that researchers have is that the cause of oral health disorders, such as these, are more complicated than a single pathogenic microbe. Both dental caries and periodontal disease have been associated with multiple microbes in the oral cavity (Jenkinson and Lamont 2005). More recently, oral bacteria have also been associated with heart disease (Joshipura et al. 1996) and even pre-term birth (Aagaard et al. 2014). Because of the associations with dental caries, oral cancer, and obesity, the oral/salivary microbiome continues to warrant further study (Lazarevic et al. 2012).

Materials and Methods

Participants, Sample and Data Collection

This research was conducted at the University of Oklahoma (OU) in the Laboratories of Molecular Anthropology and Microbiome Research (LMAMR). The Graduate College at OU facilitated this research by allowing myself contact with first year graduate students who met the qualifications for this study. Additionally, flyers (~20) were posted in public places around campus to recruit qualified participants. This research was reviewed and approved by the University of Oklahoma Institutional Review Board on May 14, 2015, and renewed April 8, 2016 (IRB# 5494).

Participants were first year graduate students at the University of Oklahoma during the Fall semester of 2015. Three males and eight females between the ages of 18-25 were enrolled (n=11); three participants (GS09-GS11) from the eleven subjects enrolled were second year graduate students, and were chosen as a comparative cohort (Table 1). Second year students were chosen on the assumption that their entry and exit samples are not expected to vary as much, as they have adjusted to graduate student life. The second year students' inclusion was a control for if and how the microbiomes vary over the course of the semester. Both Oklahoma residents and non-residents were included in the first year graduate student sample population. The second year graduate students had minimal (<1 month) to no travelling time in the summer prior to the beginning of the study (Table 1).

To analyze the changes in oral and gut microbial diversity over time, samples were collected twice. The first collection was conducted in the two weeks before the semester began (August 10, 2015 – August 27, 2015). For students arriving to Oklahoma from out of state, samples were collected within 24 hours of arrival to Norman. The timing of the sample collection was designed to capture microbial diversity before it could be overly influenced by the local environment. The second sample collection was performed in the weeks leading up to and also during final semester exams (December 7, 2015 – December 17, 2015), a time when stress may be at its highest. Anthropometric measurements (height and weight) of the participants were collected and recorded by the researcher at both sample collection periods. Additional information, including self-reported perceived stress levels, exercise habits, typical diet, and sleep behavior, was collected by the researcher during an interview. The participants were asked to rate each of these four categories on a 1 to 5 scale, 1 being an indication of the unhealthiest perceived status and a score of 5 being the healthiest perceived status. To simplify the self-reported stress data, a stressor coefficient was calculated using the following formula:

$$|\textit{sum of stress scores} - \textit{maximum stress score possible}|.$$

All stressor coefficient calculations ranged from 3 to 14 (Table 1). The stressor coefficient was then coded to *low stress* (3-6), *medium stress* (7-10), or *high stress* (11-14) stress level (Table 1). Body mass indices where calculated (BMI = [weight in pounds / (height in inches * height in inches) x

703]) and classified (< 18.5 = underweight, 18.5-24.9 = normal weight, 25-29.9 = overweight, > 30 = obese) using the height and weight data collected (Table 1 and Supplementary Table 1 & 2). All of the participants remained within their starting BMI classification for the duration of the study.

Samples

Once informed consent was obtained from the participant, instructions for sample collection were distributed. In the privacy of their home, the participants collected 5mL of saliva into a sterile vial. In addition, the subjects' freshly voided fecal samples were deposited in sterile polypropylene containers. All samples were kept on ice (<24 hours) until they could be stored in a -80°C freezer at the Laboratories of Molecular Anthropology and Microbiome Research at the University of Oklahoma. Feces and saliva samples were aliquoted to 0.25g. DNA extraction of saliva samples was conducted using the MOBIO PowerSoil DNA extraction kit according to manufacturer's instructions. DNA from fecal samples was extracted using the MOBIO protocol modified with two initial heating steps similar to those described by Obregon-Tito and colleagues (2015): heat lysis for 10 minutes at 60°C, then 10 minutes vortexing/bead beating, followed by 10 minutes at 60°C before beginning MOBIO protocol. Quantitative PRC (qPRC) was performed to determine the DNA concentration of each sample. The DNA extracts were optimized to amplify at 20 cycles.

16S rRNA gene sequencing and data processing

To characterize the taxonomic profile of the gut and oral microbiomes, the V4 hypervariable region of the bacterial 16S rRNA gene was amplified using polymerase chain reaction (PCR). Universal forward and reverse primers (515F-GTGCCAGCMGCCGCGGTAA/806R-GGACTACHVGGGTWTCTAAT) were used in addition to a unique 12bp GOLAY error-correcting barcode to multiplex the samples. PCR reactions were conducted (in triplicate with negative controls to ensure that contamination was not an issue) using Phusion Hot Start II high-fidelity DNA polymerase. PCR cycling conditions were 98°C for 30 seconds followed by 25 cycles of 98°C for 15 seconds, 52°C for 20 seconds, 72°C for 30 seconds, and a final step of 72°C for 5 minutes. Samples were pooled in equimolar amounts and sequenced on an Illumina MiSeq platform (2 X 250 bp).

The 16S rRNA gene sequence data were filtered and trimmed to remove low-quality base calls ($q < 30$), then paired reads were merged using PEAR (Zhang et al. 2014). Reads with ambiguous (N) calls were also removed prior to analysis. The paired trimmed reads were then demultiplexed, chimera filtered, and assigned to reference OTUs using *de novo* OTU picking implemented in QIIME (uclust) (Edgar 2010). Operational taxonomic units (OTUs) were clustered at 97% sequence similarity, the standard convention for species identification. The resulting OTU table was rarefied to a depth of 10,000 reads per sample and used for subsequent statistical analyses.

Alpha-diversity analyses were performed using observed species and Faith's phylogenetic diversity (PD) indices using QIIME. QIIME was also used for beta-diversity analyses which were performed using weighted UniFrac distances (Lozupone et al. 2011). The resulting distance matric was transformed using principal coordinates analysis (PCoA) and visualized. R (version 3.0.2) was used for statistical tests (corrected for multiple comparisons, fdr < 10%) and to generate boxplots (R Core Team 2013).

Comparative Data

Additional comparative sequence data were used from two microbiome studies previously conducted in and around Oklahoma by LMAMR personnel and collaborators (Obregon-Tito et al. 2015; Sankaranarayanan et al. 2015) (Table 2 and Table 3). These data were chosen because they are nearly identical in processing protocol and from individuals that share a limited geographical range, limiting sources bias. The Norman non-native control (NOR) population (n=20) from Obregon-Tito and colleagues (2015) was used, as well as the Cheyenne and Arapaho (C&A) population (n=37) from Sankaranarayanan and colleagues (2015). NOR individuals were recruited from a population of university-associated individuals. C&A individuals were recruited from the C&A tribal area. Each population provided both saliva and fecal samples. Individuals under the age of 18 were excluded. In the population comparisons performed in this study,

the graduate student samples from summer and winter have been combined to form a single population (n=22).

Graduate Student Microbiome (GSG) Participant Data

ID#	Sex	State of Origin	BMI Class	Stressor Coefficient		Stress Level	
				August	December	August	December
GS01	F	OK	Normal	4	11	Low	High
GS02	F	OK	Normal	3	7.5	Low	Medium
GS03	F	KY	Obese	11.5	11	High	High
GS04	M	MI	Normal	5	7	Low	Medium
GS05	F	OK	Under	4	6	Low	Low
GS06	M	PA	Over	7	9	Medium	Medium
GS07	F	OK	Normal	7	7	Medium	Medium
GS08	F	NM	Obese	9.5	12	Medium	High
GS09	F	OK	Normal	10.5	14	Medium	High
GS10	M	OK	Normal	6	8	Low	Medium
GS11	F	OK	Normal	6	8	Low	Medium

Table 1. Graduate students' metadata (n=11)

Non-Native Norman, OK Population – (Obregon-Tito et al. 2015)

ID #	Sample Type	Sex	Age	BMI Class
NO01	Saliva & Feces	M	20s	Norm
NO02	Saliva & Feces	F	30s	Norm
NO03	Saliva & Feces	M	40s	Norm
NO04	Saliva & Feces	M	20s	Norm
NO05	Saliva & Feces	M	20s	Norm
NO06	Saliva & Feces	M	20s	Norm
NO07	Saliva & Feces	F	30s	Norm
NO08	Saliva & Feces	F	30s	Norm
NO09	Saliva & Feces	F	30s	Norm
NO10	Saliva & Feces	M	40s	Over
NO11	Saliva & Feces	M	20s	Norm
NO12	Saliva & Feces	F	20s	Over
NO13	Saliva & Feces	M	30s	Norm
NO15	Saliva & Feces	F	50s	Over
NO16	Saliva & Feces	M	40s	Obese
NO19	Saliva & Feces	F	30s	Norm
NO20	Saliva & Feces	M	20s	Over
NO21	Saliva & Feces	M	20s	Norm
NO22	Saliva & Feces	M	20s	Obese
NO23	Saliva & Feces	F	20s	Over

Table 2. Metadata for Norman, Oklahoma samples from Obregon-Tito and colleagues (2015). Fecal samples, n=20. Saliva samples, n=20.

Cheyenne & Arapaho, OK Population - (Sankaranarayanan et al. 2015)

ID #	Sample Type	Sex	Age	Origin	BMI Class
CA01	Saliva & Feces	M	50s	Clinton	Obese
CA02	Saliva & Feces	F	50s	Clinton	Obese
CA03	Saliva & Feces	F	20s	Clinton	Obese
CA04	Saliva & Feces	M	30s	Clinton	Obese
CA05	Saliva & Feces	F	80s	Clinton	Over
CA06	Saliva & Feces	F	50s	Clinton	Obese
CA08	Saliva & Feces	F	30s	Clinton	Obese
CA09	Saliva & Feces	F	20s	Clinton	Obese
CA12	Saliva & Feces	F	40s	Concho	Obese
CA13	Saliva & Feces	F	20s	Concho	Over
CA14	Saliva & Feces	M	20s	Concho	Over
CA15	Saliva & Feces	F	30s	Geary	Over
CA16	Saliva & Feces	M	40s	Geary	Obese
CA17	Saliva & Feces	M	30s	Geary	Obese
CA18	Saliva & Feces	M	60s	Geary	Obese
CA19	Saliva & Feces	F	60s	Geary	Obese
CA20	Saliva & Feces	F	50s	Geary	Over
CA21	Saliva & Feces	F	60s	Geary	Obese
CA22	Saliva & Feces	F	40s	Geary	Over
CA23	Saliva & Feces	F	60s	Hammon	Norm
CA24	Saliva & Feces	M	50s	Hammon	Obese
CA25	Saliva & Feces	M	60s	Hammon	Obese
CA26	Saliva & Feces	M	50s	Hammon	Obese
CA27	Saliva & Feces	M	50s	Hammon	Obese
CA28	Saliva & Feces	F	50s	Hammon	Obese
CA29	Saliva & Feces	F	40s	Hammon	Norm
CA30	Saliva & Feces	M	50s	Hammon	Obese
CA31	Saliva & Feces	M	60s	Hammon	Obese
CA32	Saliva & Feces	F	40s	Hammon	Obese
CA33	Saliva & Feces	F	60s	Kingfisher	Obese
CA34	Saliva & Feces	F	40s	Kingfisher	Obese
CA35	Saliva & Feces	F	40s	Kingfisher	Over
CA36	Saliva & Feces	M	60s	Kingfisher	Over
CA37	Saliva & Feces	F	50s	Kingfisher	Obese
CA38	Saliva & Feces	F	20s	Kingfisher	Obese
CA39	Saliva & Feces	F	20s	Kingfisher	Obese
CA40	Saliva & Feces	M	50s	Kingfisher	Obese
CA41	Saliva & Feces	F	50s	Kingfisher	Obese

Table 3. Metadata for Cheyenne and Arapaho samples from Sankaranarayanan and colleagues (2015). Fecal samples, n=37. Saliva samples, n=37

Results

Graduate Students' Microbiome analysis

The human microbiome is a dynamic and complex system of organisms that is under the constant influence and stress of our daily lives and environment. The oral and gut microbiomes of first-year graduate students sampled in this study did display changes in community structure, as might be expected. However, the changes between the sampling periods were largely not statistically significant due largely in part to the low sample size. To increase the power of the statistical tests similar data were added for comparison (Obregon-Tito et al. 2015; Sankaranarayanan et al. 2015).

The graduate students' oral microbiomes were dominated by the phyla *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia,* and *Tenericutes* (Figure 1a), which have been previously described in oral microbial studies (Costalonga and Herzberg 2014; Dewhirst et al. 2010; He and Shi 2009). The level of *Proteobacteria* increases in all participants, with the exception of GS01.

Similarly, the graduate students' gut microbiomes were also dominated by the phyla *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia,* and *Tenericutes* (Figure 1b), though in expectedly different frequencies. The changes in the graduate students' gut microbiomes are primarily noticeable in the abundance of *Firmicutes* and *Bacteroidetes,* both of which dominate the samples (Figure

1b). The first-year graduate students (GS01-GS08) all display changes in gut microbiome community structure without any evident trends. The gut microbiome of GS06 is dominated by *Firmicutes* (>91%) and changed least among first-year participants between sampling periods.

This analysis finds that a family within the *Firmicutes* phylum known as *Ruminococcaceae* exhibits a statistically significant ($p < 0.05$, fdr < 0.1) increase in abundance in participants with BMI < 25 (under weight and normal weight classifications) between the sampling periods. The average abundance of *Ruminococcaceae* more than doubled (~135%) in the graduate students' gut microbiomes from summer to winter (Figure 2).

Biodiversity is measured and reported in microbiome studies through two metrics, alpha- and beta-diversities, both of which will be discussed below following a brief description of their scope. Alpha-diversity is a measurement that can be estimated by either species richness or Faith's Phylogenetic Diversity. In microbiome studies, such as this, species richness, or the number of species in a sample, are described in operational taxonomic units (OTUs). Operation taxonomic units are the preferred measurement over "species" because 16S rRNA sequences do not perfectly correspond to what might be thought of as a species. OTUs are defined by 97% sequence similarity, and as such, are more objective than alternate species definitions. Alpha-diversity is reported here by both observed operational taxonomic units and Faith's Phylogenetic Diversity (PD) for both the oral and fecal samples (Figure 3). Beta-diversity compares the individuals' microbiome communities

to each other to understand the similarity or dissimilarity of the communities' structure (Figures 4, 5, & 6).

Our alpha-diversity analysis exhibits no statistically significant changes in the graduate students' gut or oral microbiomes between the summer and winter sampling periods (Figure 3). When beta-diversity of the graduate students' gut microbiomes is graphically depicted, personal variation can be seen (Figure 4). The amount of personal variation seen between sampling points is generally consistent across the majority of both the in- and out of state students'. A single out of state student (GS06) displays minimal change between the sampling points, similar to that of two of the second year comparative graduate students. One in-state student (GS09) exhibits a large range of variation between summer and winter.

When analyzing beta-diversity by season, no consistent patterns were evident (Figure 5a). Stress level classification also seems to not correlate to the graduate students' changes in microbial community structure (Figure 5c). However, these factors of influence cannot be ruled out.

Population comparison analysis

The NOR population has been previously reported to have increased oral microbial richness when compared to the C&A population (Ozga et al. 2016) and I find this result to remain true (Figure 3a, b). The range of diversity in the C&A individuals is wider than that of the NOR individuals,

but the average alpha-diversity of the C&A individuals is lower. Intriguingly, I find that the C&A oral microbiome is more similar to that of the graduate students' microbiome of this study at either sampling point (Figure 3a, b) than it is to the NOR population.

The graduate student gut data at both sampling points show a slight increased richness when compared to the other studies' populations, though not significantly (Figure 3c, d). When compared to South American traditional and rural societies, it was reported that the gut microbiomes of the NOR and C&A participants also display a significant decreased microbial richness (Obregon-Tito et al. 2015; Sankaranarayanan et al. 2015).

The analysis of gut microbial beta-diversity by geography shows minimal variation between populations (Figure 5b). The graduate students display only a small visual indication of an association attributable to their out-of-state status (Figure 4 & 5b), though the variation of the graduate student population as a whole is higher than either the NOR or C&A individuals. Overall, each population's beta-diversity is nested within one another, with the graduate students' beta-diversities showing the widest range of variation along PC1 (Figure 5d).

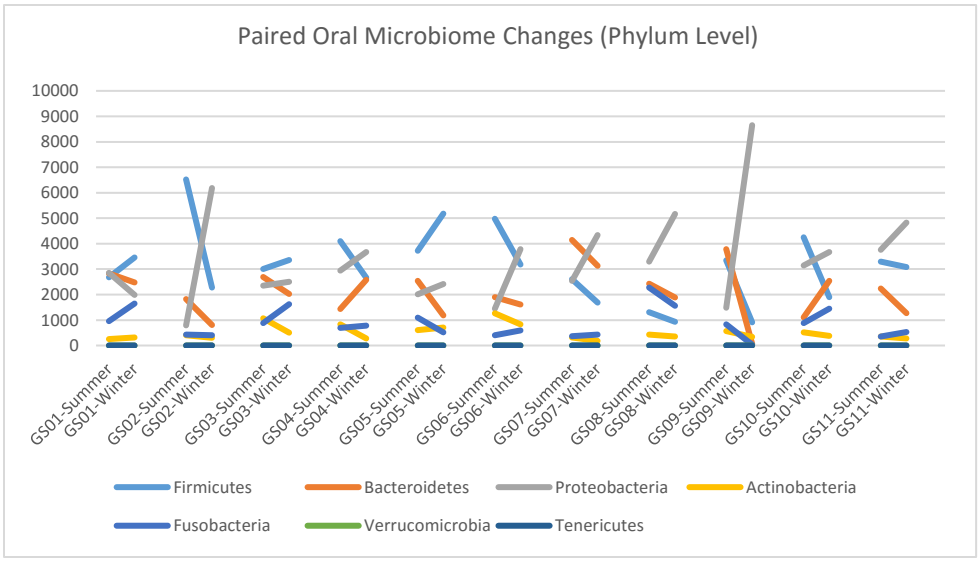
The analysis by age shows an underlying influence on the beta-diversity between the samples; the oldest participants' samples converge toward each other and those younger participants' samples displaying more variation (Figure 6).

In the oral microbiomes, the genera *Actinomyces*, *Haemophilus,* and *Prevotella* are noticeably enriched in these populations. The graduate student population mean abundance of *Actinomyces* decreases (phylum: *Actinobacteria* [Figure 1a]) to resemble that of the C&A (Figure 7a). Interestingly, the NOR are significantly (p = 3.20 E -09, fdr = 1.13 E -05) enriched in this same genus (Figure 7a). *Haemophilus* levels of abundance in the graduate students is high and increases slightly between sampling periods (Phylum: *Proteobacteria* [Figure 1a]). As a population, the graduate students' samples are significantly (p = 3.56 e -06, fdr = 0.002) enriched in *Haemophilus* compared to the C&A and NOR (Figure 7b). *Prevotella* is enriched in the C&A (Figure 7c), though not significantly (p = 0.06, fdr = 1). The mean abundance of *Prevotella* decrease in the graduate student population (-66%) between sampling periods (Phylum: *Bacteroidetes* [Figure 1a]).

The gut microbiomes also exhibit interesting comparative results at the genus level among the taxa *Bacteroides* and *Blautia.* The graduate student population has a significantly (p = 1.14 E -06, fdr = 0.0013) higher abundance of *Bacteroides* than the C&A or NOR populations (Figure 8a). The differences in abundance of *Blautia* is significantly (p = 6.63 E -05, fdr = 0.025) enriched in the C&A samples with the graduate students' low level of abundance of the genus resembling that of the NOR (Figure 8b). The graduate students mean abundance did increase between the two sampling periods, but the increase was not enough to be seen in the population comparison.

The population comparison also yields interesting results at the family level, that show significant differences between the graduate students and the other two populations. Differences in *Ruminococcaceae*, discussed above (Figure 2), narrowly missed the significance threshold during the population level comparison (p = 0.00075, fdr = 0.143), but remain interesting for the high frequency of the taxa (Figure 9a) in comparison to the other Oklahoma populations. The differences in frequency of *Erysipelotrichaceae* and *Lachnospiraceae* between the populations are also significant (Figure 9b, c), though minimal (p = 4.29 E -07, fdr = 0.00075; p = 0.00028, fdr = 0.072, respectively).

a.



b.

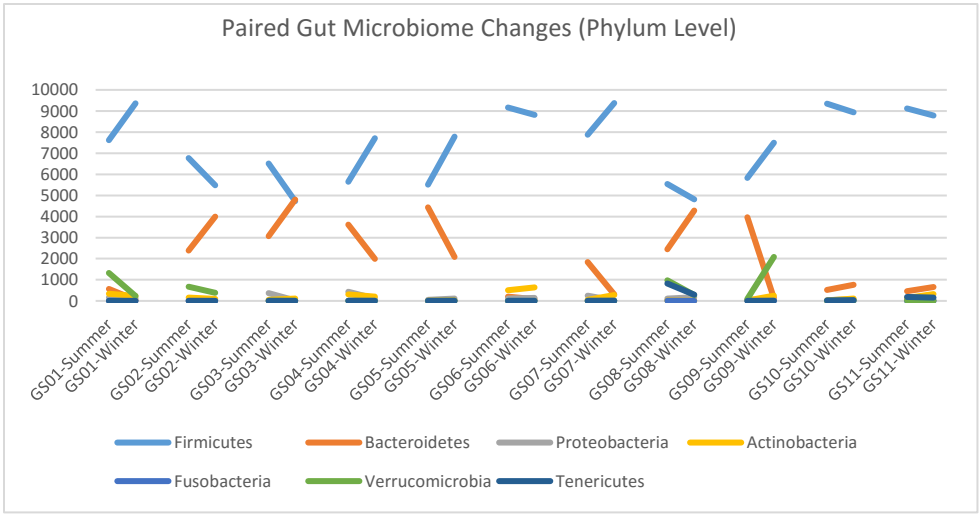


Figure 1. Phylum level changes in graduate students' microbiomes.
a) Oral microbiome phylum level changes. b) Gut microbiome phylum level changes. The students' paired samples are shown with colored lines representing each phyla, the slope of the line indicates increase or decrease of that particular phyla.

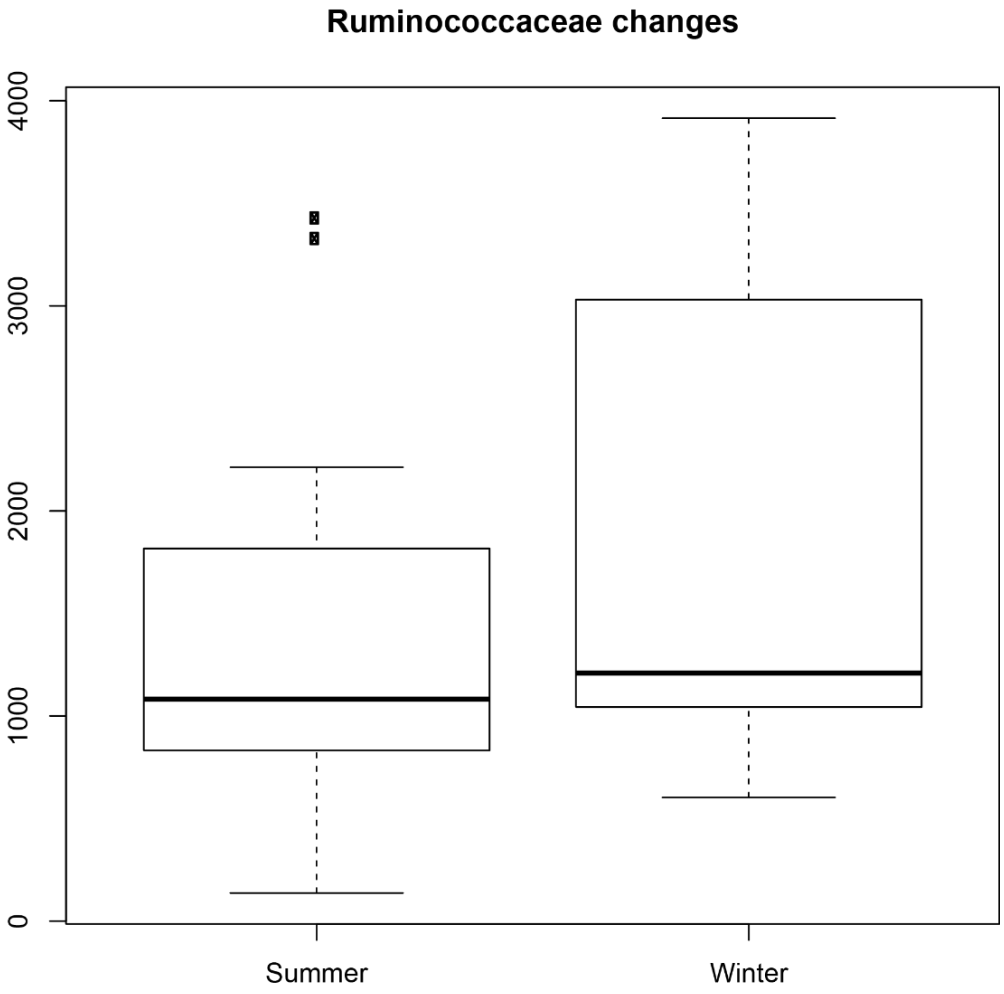


Figure 2. Changes in graduate students' gut microbe *Ruminococcaceae* between summer and winter. This result becomes significant when the population is compared based on BMI (p = 0.003, fdr = 0.08). *Ruminococcaceae* is enriched in individuals with lower BMI.

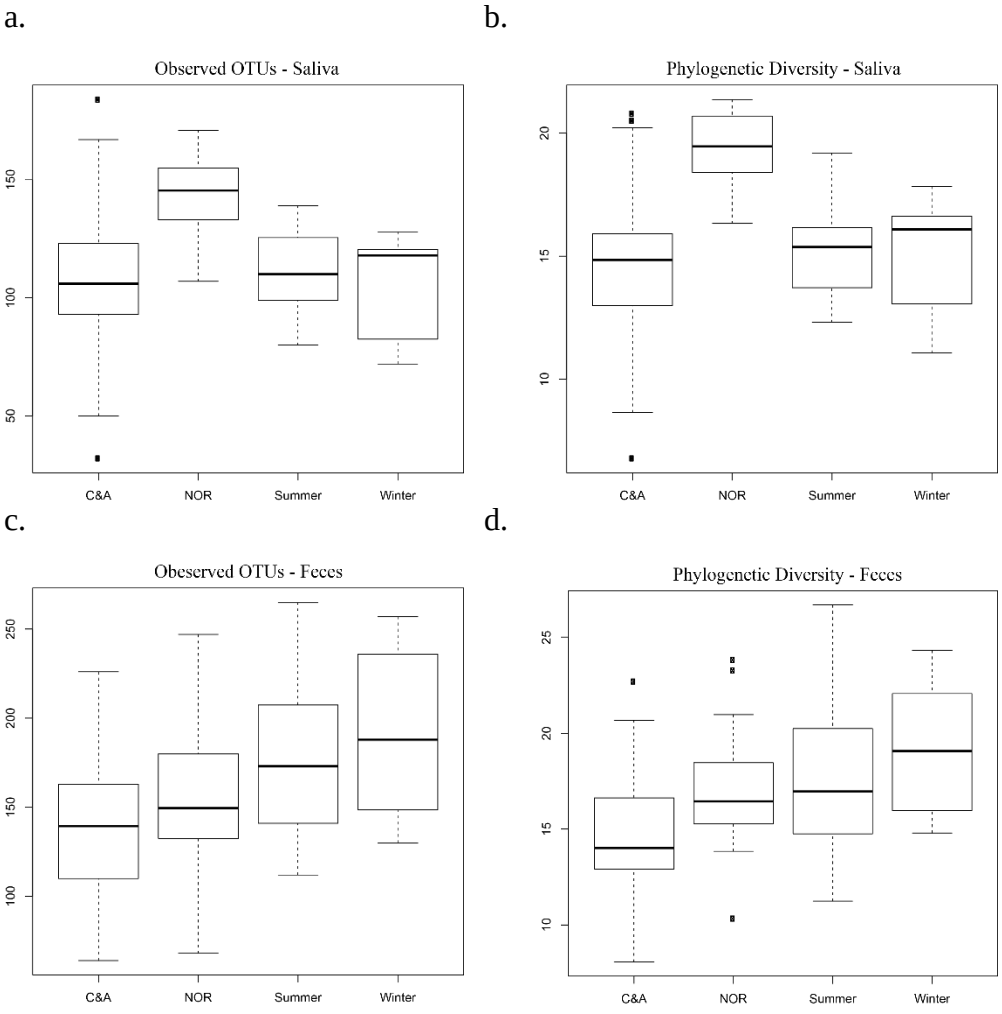


Figure 3. Alpha-diversity comparisons. a) Oral microbial richness measured by observed OTUs. b) Oral microbial richness measured by phylogenetic diversity. c) Gut microbial richness measured by observed OTUs. d) Gut microbial richness measured by phylogenetic diversity. Comparative data from Oklahoma studies added; C&A – Cheyenne and Arapaho individuals of Oklahoma (Sankaranarayanan et al. 2015), NOR – Non-native Norman, Oklahoma individuals (Obregon-Tito et al. 2015). Saliva data show little change from sample time points. Fecal data show a slight increase in alpha diversity.

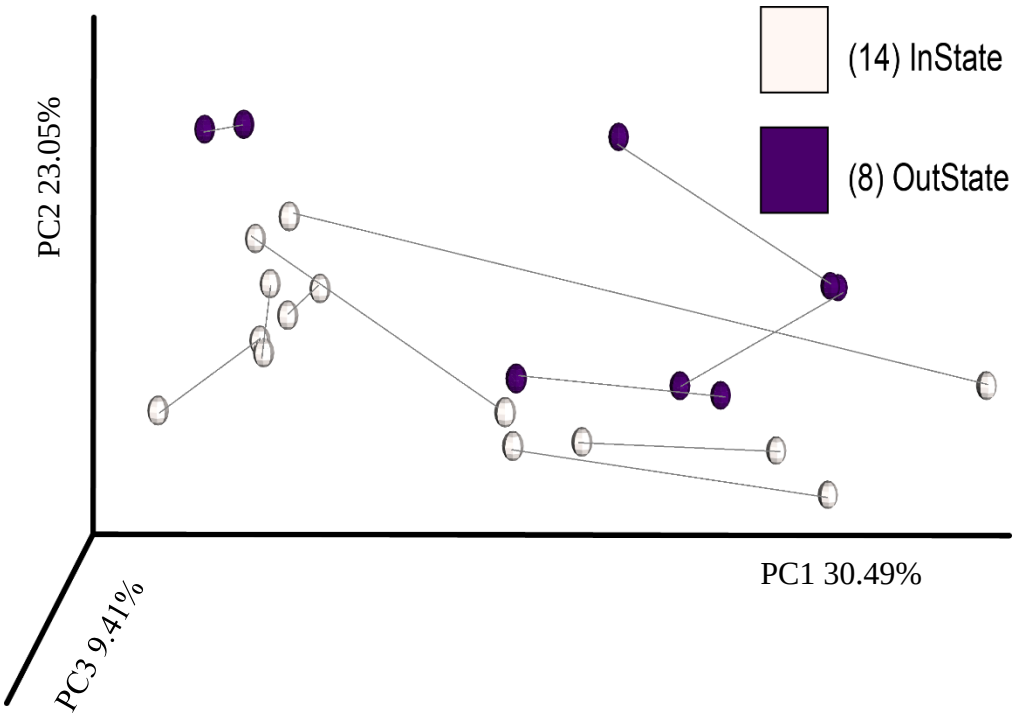


Figure 4. Graduate student beta-diversity by geography. The graduate students' beta-diversity is depicted. Lines connect the paired samples of each individual's summer and winter data**.** The change seen, represented by the length of the lines, is shorter than what would be expected if the samples were distributed randomly in the plot.

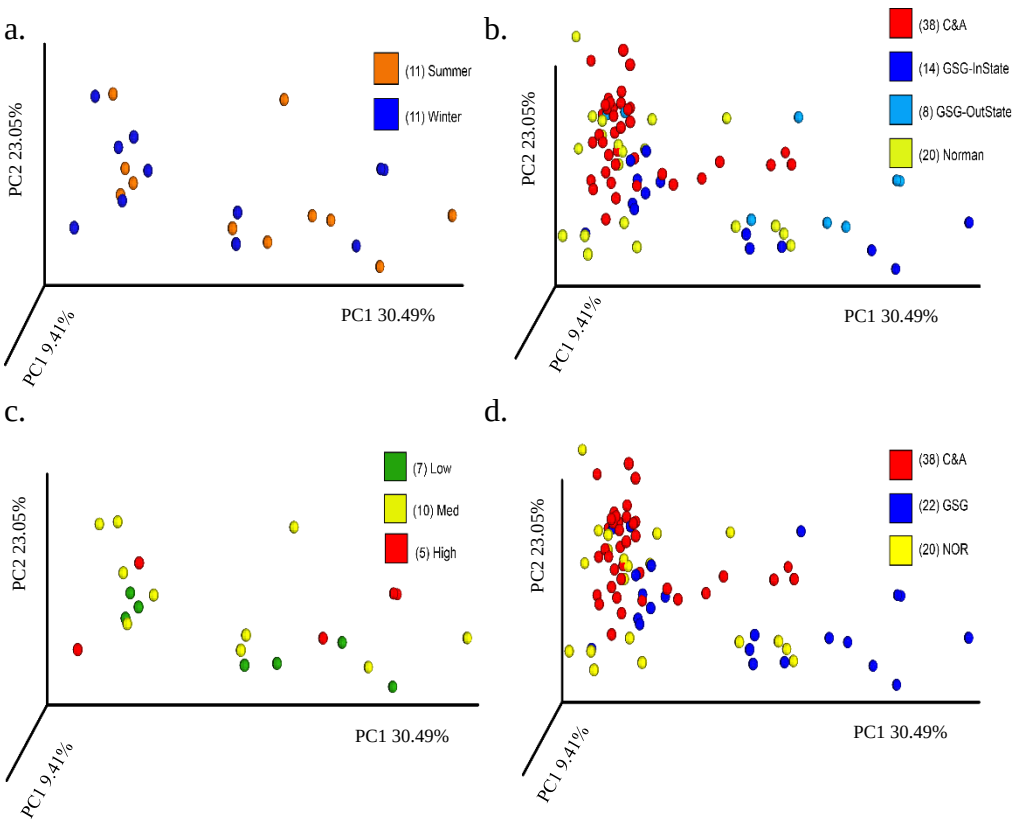


Figure 5. Beta-diversity comparisons of gut microbiomes. a) Graduate student population by sampling period. b) Multiple study population by geography. c) Graduate student population by stressor coefficient level. d) Multiple study comparison. Proportion of variance explained by each principal component axis is denoted in the corresponding axis label.

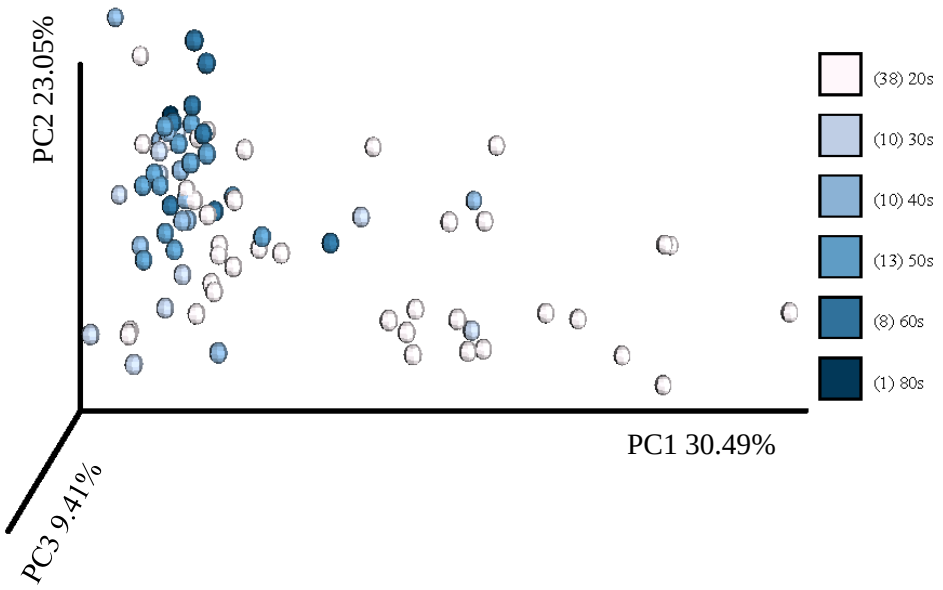


Figure 6. Beta-diversity comparison across populations (C&A, GSG, and NOR) by age. Older study participants appear to converge toward the left of the plot. Younger participants show a wide range of variation in their beta-diversity. Age can be seen to be an influential factor, though not the key factor driving diversity.

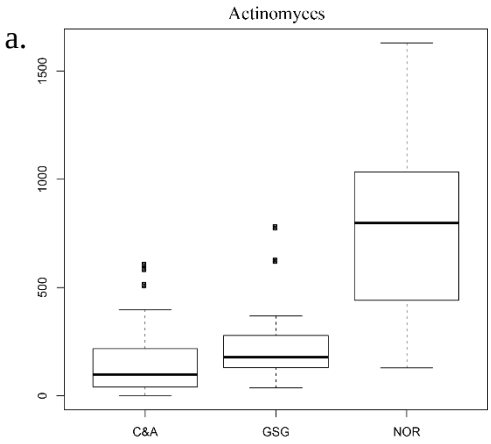
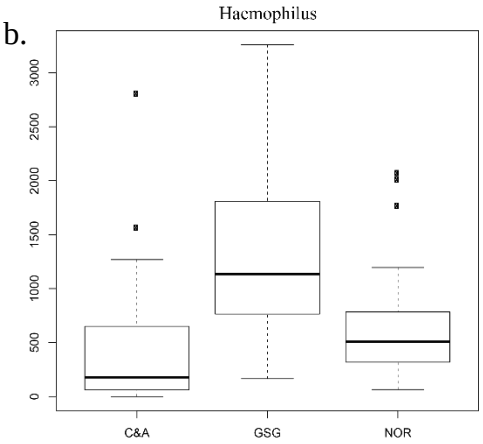
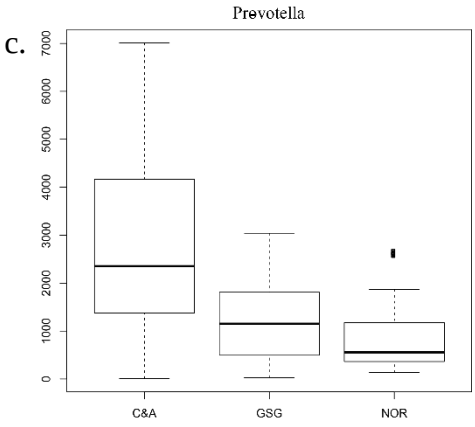
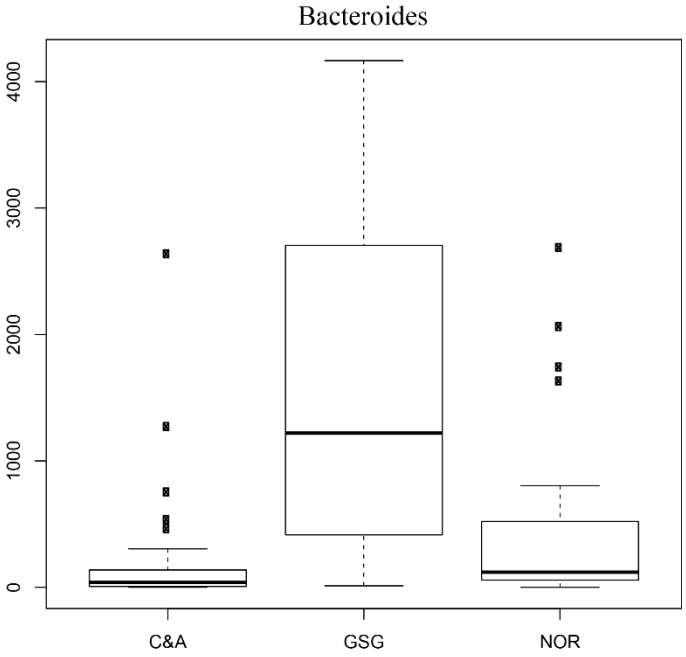


Figure 7. Boxplots of relative abundance of oral taxa (genus level). a) *Actinomyces* is enriched in NOR. b) *Haemophilus* is enriched in the graduate students combined season samples (n=24). c) *Prevotella* is enriched in the C&A.





a.



b.

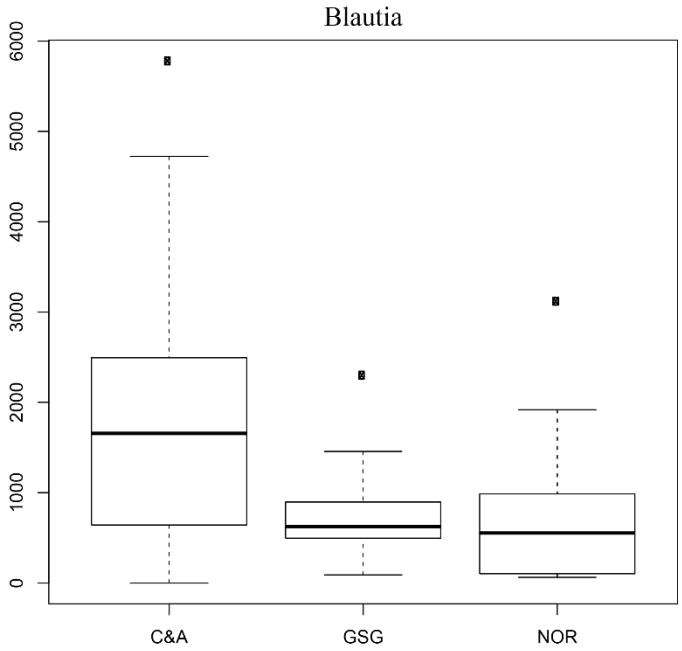
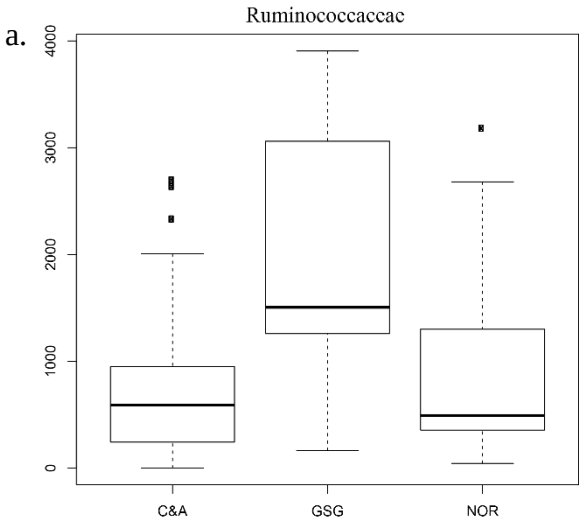
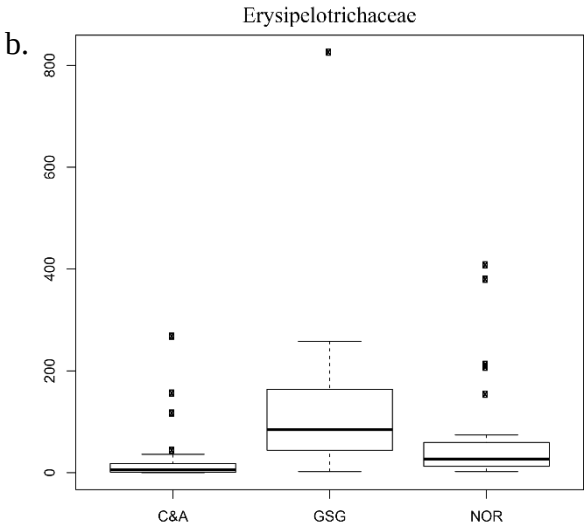


Figure 8. Boxplots of relative abundance of gut taxa (genus level). a) *Bacteroides* is at higher levels in the graduate student population's combined season samples b) *Blautia* frequency is highest in the C&A. The GSG combined season samples resemble the level of abundance in the NOR population.

a.



b.



c.

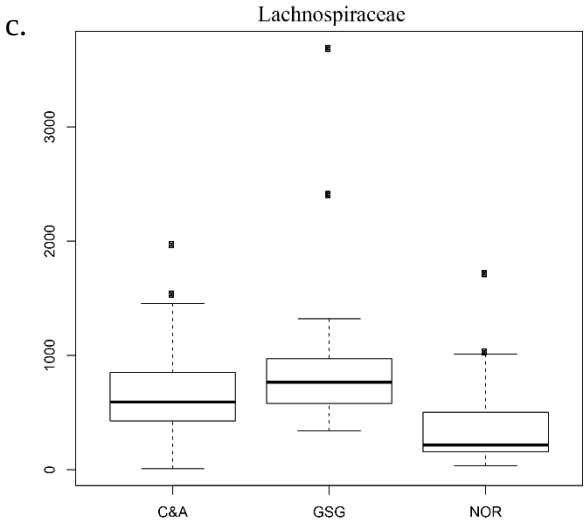


Figure 9. Boxplots of relative abundance of taxa (family level). GSG samples from summer and winter combined to form a single comparative population.
a) *Ruminococcaceae* is enriched in the graduate student population.
b) *Erysipelotrichaceae* is enriched in the graduate student population.
c) *Lachnospiraceae* is enriched in the graduate student population.

Discussion

The goal of this study was to characterize the change in the microbiomes of graduate students in order to understand if stress and the impact of relocation is evident in the microbial community structure of the study participants. The results confirm that change is inherent to the microbiome and that the microbiome's ability to adapt can be recorded. However, no significant findings could be made to directly implicate any one factor as a key cause of the microbiome changes. Stress or the impact of relocation cannot, at this time, be ruled out. Rather than finding stress or relocation to be directly associated with change in microbial community structure, there is an observed influence based on age. Additionally, at this time the possibility of seasonality also playing an influential role in microbiome structure cannot be ruled out.

The slight increase in gut microbial diversity (Figure 3c, d) could be attributable to changes in the participants' diets. If dietary changes are the cause of the changes seen in the microbiome data, they would need to be disentangled from other factors, for example, the consumption of different regionally available foods, or the possibility of unhealthy stress eating. Natural seasonal climate change can also impact the environmental microorganisms the body comes into contact with (Bowers et al. 2011) and ultimately consumes, this can have an impact on our microbiomes structure. This same seasonal shift may also play a role in the availability of a variety of

fresh food sources and could result in changes in microbial diversity (Davenport et al. 2014). In this case, a detailed dietary log would be beneficial for this analysis, but was not performed for this study.

The slight increase shown in alpha-diversity in the graduate students is similar to that reported by Davenport and colleagues (2014) in their Hutterite population and under similar seasonal conditions (summer vs. winter). Unlike the Davenport et al. (2014) study, the participants in this study did not provide detailed food logs. Additionally, the graduate population was not as uniform as the Hutterite population. This begs the question of whether we should expect to see even more increased diversity from summer to winter, had the population been more uniformly controlled.

The oral microbiome results of this study necessitate further investigation. The increase in alpha-diversity of the graduate students' oral microbiome was only slight (Figure 3a, b). Additionally, the level of alpha-diversity resembled that of the C&A population and not the NOR samples. I would have expected the graduate students' oral microbial structure to come to reflect that of the NOR individuals because they are geographically similar. Both the graduate students and the NOR population are primarily located in Norman, Oklahoma; whereas, the C&A are more isolated in rural Oklahoma. I suspect that the increased frequency that I see in *Actinomyces* in the NOR population is driving this difference. *Actinomyces* is a primary colonizer of the oral cavity and is part of the foundation of forming biofilm in the mouth.

Further analysis will need to be conducted in order to rule out other possible microbial associations.

This project attempted to characterize factors of stress, such as diet, geography, or behavioral changes that might be evident in the microbiome. These multiple factors become confounding factors in the small sample size provided here. It is this fact which led these researchers to calculate the stressor coefficient. However, as can be seen here and in other studies (Marzorati et al. 2016), stress to the microbiome comes in many forms and is experienced by the individual on a personal level.

This project has provided several considerations for future research. This same study would benefit and would yield more precise results by controlling for a single stress factor to investigate. I found the self-reporting of stress to be too interpersonal and indefinable. This population of graduate students came from several fields of expertise and it became apparent that the expectations of each academic department are tailored to that field. For example, a Health and Exercise Science student is likely experiencing different demands on his/her body's biology than a student studying Chinese Art History. Using an assay designed for stress may allow for results that could be more directly associated to types and levels of stress. One possible option may be to test levels of cortisol contained in the saliva samples as an indication of stress level.

A standardized food log or food recall survey, in addition to multiple sampling points would be most ideal for associating changes in microbial

structure to diet rather than to season. Multiple sampling points throughout the study may highlight patterns of seasonality. Seasonality as a factor in microbiome influence is discussed in microbiome studies (Bowers et al. 2011; Davenport et al. 2014), but it is possible that its effects are not always taken into consideration when designing studies or making population comparisons. This begs the question of whether seasonality is biasing microbiome study results. The above study design modifications and consideration will lead future research to more relevant indications of change in microbial community structure.

I find that the microbiomes of this study population changed between the two sampling periods. However, the directionality of these changes is not consistent across individuals, and at present, it is not possible to disentangle which factors may be influencing these changes. I show that seasonality and age are additional candidates of influence to these human gut and oral microbial ecologies. However, the factors that carry the most influence are difficult to pinpoint. In all likelihood it is several factors causing this change and future work will address these factors of influence more precisely. I continue to believe that graduate students are ideal participants for a study like this. These results shed light on further research designs and raise questions about the timing of study implementation and sampling. It is studies such as this one that help establish associations to the changes recorded in human microbiomes. Through these associations, hypotheses can be made with the hopes of arriving to an understanding, intervention, or possible treatment.

Though this study did not result in conclusive findings, I have presented work that profiles human microbiome structure and change. Science must continue to investigate the combined abilities encoded within our genes and the genes of our microbial commensals in order to provide humankind with information on possible treatments when we encounter maladaptive microorganisms and/or situations.

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Appendix I: Supplementary Tables

Graduate Student Microbiome (GSG) participant survey data

ID#	Sleep		Exercise		Diet		Stress	
	Aug	Dec	Aug	Dec	Aug	Dec	Aug	Dec
GS01	4	3	4	2	3	2	5	2
GS02	3	2	5	4	4	4.5	5	2
GS03	3	3	1	1	2.5	3	2	2
GS04	3	3	4	4	4	4	4	2
GS05	4	3	3	3	4	5	5	3
GS06	3	2	4	4	4	3	2	2
GS07	2	1	3.5	4	1	3	4.5	5
GS08	3	2	1	1	2.5	3	4	2
GS09	4	2	1	1	2.5	2	2	1
GS10	3	3	5	3	4	4	2	2
GS11	4	3	3	1	4	4	3	4

Table S1. Self-reported perceived stress levels, exercise habits, typical diet, and sleep behavior. The participants were asked to rate each of the above categories on a 1 to 5 scale, 1 being an indication of the *unhealthiest* perceived status and a score of 5 being the *healthiest* perceived status.

Graduate Student Microbiome (GSG) biometric data

ID#	Height	Weight (lbs.)		BMI	
		Aug	Dec	Aug	Dec
GS01	5' 10.5"	167.5	164.4	23.7	23.3
GS02	5' 6"	140.6	147.4	22.7	23.8
GS03	5' 3"	173.4	173.4	30.7	30.7
GS04	5' 7"	146.2	147	22.9	23
GS05	5' 3"	99	97.2	17.5	17.2
GS06	5' 8.5"	187.5	185.8	28.1	27.8
GS07	5' 7"	149	154	23.3	24.1
GS08	5' 7.5"	204	202	31.5	31.2
GS09	5' 11"	159.6	155.8	22.3	21.7
GS10	6' 0"	167	166.2	22.6	22.5
GS11	5' 6"	147.8	148	23.9	23.9

Table S2. Body mass indices where calculated (BMI = [weight in pounds / (height in inches * height in inches) x 703]) and classified (< 18.5 = underweight, 18.5-24.9 = normal weight, 25-29.9 = overweight, > 30 = obese) using the height and weight data collected and reported above. All of the participants remained within their starting BMI classification for the duration of the study.

Norman, OK (NOR) control population metadata - Obregon-Tito et al. (2015)

ID#	Age	Sex	BMI	BMI Class
NO01	23	M	21.69	Norm
NO02	37	F	20.52	Norm
NO03	40	M	23.37	Norm
NO04	26	M	24.16	Norm
NO05	28	M	22.19	Norm
NO06	28	M	23.49	Norm
NO07	32	F	21.92	Norm
NO08	32	F	20.01	Norm
NO09	34	F	23.77	Norm
NO10	41	M	26.58	Over
NO11	26	M	23.93	Norm
NO12	27	F	28.62	Over
NO13	35	M	20.34	Norm
N015	50	F	25.92	Over
NO16	47	M	30.86	Obese
NO19	32	F	19.3	Norm
NO20	26	M	27.86	Over
NO21	23	M	24.78	Norm
NO22	26	M	30.22	Obese
NO23	26	F	26.53	Over

Table S3. Data incorporated for comparison. Data reported on the control participants of Obregon-Tito et al. (2015).

Cheyenne & Arapaho, OK (C&A) population metadata –

Sankaranarayanan et al. (2015)

ID#	Sex	Age	BMI
CA01	M	55	31
CA02	F	55	40.8
CA03	F	27	35
CA04	M	30	33.1
CA05	F	84	27.3
CA06	F	51	44.2
CA08	F	33	38
CA09	F	29	48.3
CA12	F	43	32.3
CA13	F	20	27.3
CA14	M	21	25.2
CA15	F	34	28.2
CA16	M	45	39.1
CA17	M	39	40.3
CA18	M	69	36.3
CA19	F	68	40.3
CA20	F	54	26.1
CA21	F	65	40.7
CA22	F	41	26.2
CA23	F	65	24.7
CA24	M	55	31.8
CA25	M	65	30
CA26	M	56	42.1
CA27	M	55	44
CA28	F	55	36.3
CA29	F	45	24.9
CA30	M	55	37.4
CA31	M	62	30
CA32	F	44	43.7
CA33	F	69	35
CA34	F	44	33.4
CA35	F	49	28
CA36	M	66	29.6
CA37	F	50	32.6
CA38	F	29	47.4
CA39	F	29	31.9
CA40	M	55	35.1
CA41	F	55	36.1

Table S4. Data incorporated for comparison. Data reported on the C&A participants of Sankaranarayanan et al. (2015).

Appendix II: Supporting Documents

IRB Approval Letters

The UNIVERSITY of OKLAHOMA

Institutional Review Board for the Protection of Human Subjects

Approval of Study Modification – Expedited Review – AP0

Date: July 21, 2015 **IRB#:** 5494

Principal Investigator: Justin Roy Lund **Reference No: 641807**

Study Title: Characterizing microbiome changes in the graduate student gut

Approval Date: 07/21/2015

Modification Description:
Recruitment material modified.

The review and approval of this submission is based on the determination that the study, as amended, will continue to be conducted in a manner consistent with the requirements of 45 CFR 46.

To view the approved documents for this submission, open this study from the My Studies option, go to Submission History, go to Completed Submissions tab and then click the Details icon.

If the consent form(s) were revised as a part of this modification, discontinue use of all previous versions of the consent form.

If you have questions about this notification or using iRIS, contact the HRPP office at (405) 325-8110 or irb@ou.edu. The HRPP Administrator assigned for this submission: Karen Braswell.

Cordially,

E. Laurette Taylor, Ph.D.
Chair, Institutional Review Board

The UNIVERSITY of OKLAHOMA

Institutional Review Board for the Protection of Human Subjects

Approval of Continuing Review – Expedited Review – AP0

Date: April 08, 2016 **IRB#:** 5494

Principal Investigator: Justin Roy Lund **Approval Date:** 04/08/2016 **Expiration Date: 03/31/2017**

Expedited Category: 3

Study Title: Characterizing microbiome changes in the graduate student gut

Based on the information submitted, your study is currently: Active, closed to enrollment. On behalf the Institutional Review Board (IRB), I have reviewed and approved your continuing review application. To view the documents approved for this submission, open this study from the *My Studies* option, go to *Submission History*, go to *Completed Submissions* tab and then click the *Details* icon.

As principal investigator of this research study, you are responsible to:

- Conduct the research study in a manner consistent with the requirements of the IRB and federal regulations 45 CFR 46.
- Obtain informed consent and research privacy authorization using the currently approved, stamped forms and retain all original, signed forms, if applicable.
- Request approval from the IRB prior to implementing any/all modifications.
- Promptly report to the IRB any harm experienced by a participant that is both unanticipated and related per IRB policy.
- Maintain accurate and complete study records for evaluation by the HRPP Quality Improvement Program and, if applicable, inspection by regulatory agencies and/or the study sponsor.
- Promptly submit continuing review documents to the IRB upon notification approximately 60 days prior to the expiration date indicated above.
- Submit a final closure report at the completion of the project.

You will receive notification approximately 60 days prior to the expiration date noted above. You are responsible for submitting continuing review documents in a timely fashion in order to maintain continued IRB approval.

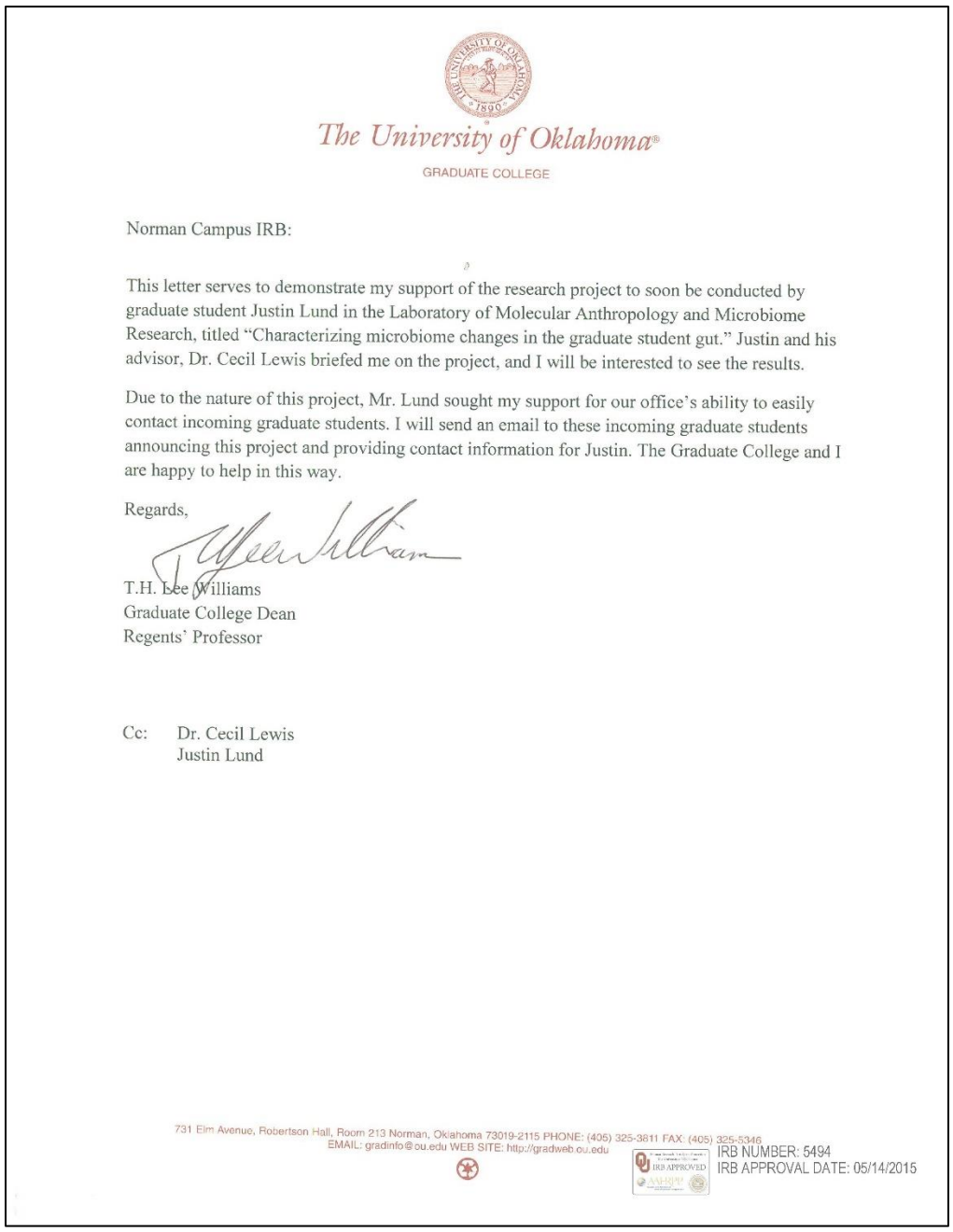
If you have questions about this notification or using iRIS, contact the IRB @ 405-325-8110 or irb@ou.edu.

Cordially,

E. Laurette Taylor

E. Laurette Taylor, Ph.D.
Chair, Institutional Review Board

Graduate College Letter of Support



The University of Oklahoma

GRADUATE COLLEGE

Norman Campus IRB:

This letter serves to demonstrate my support of the research project to soon be conducted by graduate student Justin Lund in the Laboratory of Molecular Anthropology and Microbiome Research, titled "Characterizing microbiome changes in the graduate student gut." Justin and his advisor, Dr. Cecil Lewis briefed me on the project, and I will be interested to see the results.

Due to the nature of this project, Mr. Lund sought my support for our office's ability to easily contact incoming graduate students. I will send an email to these incoming graduate students announcing this project and providing contact information for Justin. The Graduate College and I are happy to help in this way.

Regards,

T.H. Lee Williams
Graduate College Dean
Regents' Professor

Cc: Dr. Cecil Lewis
Justin Lund

731 Elm Avenue, Robertson Hall, Room 213 Norman, Oklahoma 73019-2115 PHONE: (405) 325-3811 FAX: (405) 325-5346
EMAIL: gradinfo@ou.edu WEB SITE: http://gradweb.ou.edu

IRB APPROVED
IRB NUMBER: 5494
IRB APPROVAL DATE: 05/14/2015

Recruitment Flyer

