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Bacteria's Best Friend: Factors that Contribute to Oral Microbiome Change in the Domesticated Dog and its Effects on Human Relationships

LAUREN STROUPE
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Reviewed and approved* by the following:

Laura Weyrich
Professor of Anthropology and Bioethics
Thesis Supervisor

Doug Bird
Professor of Anthropology
Honors Advisor

* Electronic approvals are on file.

ABSTRACT

Dogs are an integral part of modern society and of the past. Our society values dogs in many ways, from service, hunting, farming and family pets. While humans and dogs are not genetically close, they share a unique history of proximity. Archaeological and molecular data has shown evidence of cohabitation for thousands of years. This relationship has impacted the health of dogs and humans. Bacteria can help understand the impacts of this relationship. Recent research on the microbiome – the diverse communities of bacteria, viruses, and eukaryotes – has furthered our understanding of relationships between humans and dogs. However, the oral microbiome in dogs has been understudied. This relationship warrants more research on the anthropological relationship of dog and human oral microbiota. While there are differences in the oral microbiome of dogs and humans, dogs share an environment and history with humans and therefore can be a relevant proxy on human relationships. This paper will explore the current literature of human and dog oral microbiomes. I present and test hypotheses based on the possible factors contributing to changes in the dog oral microbiome and how those factors can be seen in the human oral microbiome. The results from these hypotheses are that only dog age contributed to a statistical significance of oral bacterial composition difference in the dog sample. Park frequency, sex and other pets in failed to reject the null hypotheses. Future research considerations will be presented to further understand the oral microbiota of dogs and its impact on humans.

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Finally, for Dexter and Blaze, this one's for you woman's best friend!

INTRODUCTION

It is important in the field of anthropology to understand the effects humans have had on dogs because for thousands of years, dogs and humans have created a shared environment and these adaptations have affected dogs' behavior and biology (Axelsson et al, 2013; Coelho et al.,2013; Salomons et al., 2021). Past studies have revealed that humans have shaped the social and cognitive behaviors of dogs creating a tamer and more communicative animal to be able to live among humans (Zeder, 2012). The communicative differences between dogs and wolves have been studied and reveals that dogs are better able to understand human gestures (Topal et al.,2009). Dogs and humans share similar social behaviors that resulted in similar evolution and development by coexisting with humans (Topal et al., 2009). Previous research has revealed that there is DNA difference in dogs and wolves (Cagan &Blass, 2016) along with different bacteria diversity present in the oral microbiome of dogs and wolves (Wu et al, 2017); therefore, could humans have affected the oral microbiome of dogs? This paper will examine the current literature to better understand the context of the composition and changes of the human oral microbiome, a novel mechanism that can mediate human and dog interactions and potential behavior. Hypotheses based on the data collected from Tudek Dog Park in State College will be analyzed to determine whether certain factors have an impact on the oral bacteria of the dog cohort.

Human Oral Microbiome:

The health of an organism relies on the symbiosis of the host and the bacteria that inhabit it (Ghaisas et al., 2016). The gut microbiome has substantial impacts on the health of organisms (Zaura et al., 2009). Health related diseases such as obesity, Alzheimer's, and Parkinson's are related to the gut microbiome (Turnbaugh et al.,2009; Peña et al., 2017, Wu et al., 2021). Neurological diseases have also been linked to disruptions in the gut microbiome (Ghaisas et al., 2016). While research on the impact of bacteria onto host health has primarily been on the gut microbiome, there is growing evidence that the oral microbiome also impacts health (Kleinstein et al., 2020). The two most common oral diseases are caries and periodontitis (Pritchard et

al.,2017; Van der Velden et al., 1986). While these diseases affect the oral cavity, they can also increase risk for systemic disease (Daspher et al., 2019).

Humans and bacteria have a long and complicated history (Baker & Edlund, 2019). It is theorized that ancient microbiota was more diverse than present human microbiota (Adler et al., 2013; Weyrich,2021). The development of the human oral microbiome begins in early life (Dzidic et al., 2018). The human oral microbiome is influenced by the mother and begins to develop with pioneer bacteria such as *Streptococcus* and *Actiomyces* at birth (Dzidic et al., 2018). The oral microbiome is not stable until the arrival of the permanent dentition and continues to be shaped by the environment and transmission by the parents (Dzidic et al., 2018). While there is diversity of bacteria in the oral microbiome due to shedding and solid surfaces of the oral cavity (Zaura et al., 2009), the oral microbiome is relatively stable to colonization of foreign pathogens (Baker & Edlund, 2019). The human oral microbiota composition depends on the location in the oral cavity (Segata et al., 2012). Certain bacteria colonize only parts of the oral cavity (Keijser et al., 2008). *Actinobacteria* and *Fusobacteria* are present on dental plaque in humans (Keijser et al., 2008; Davis et al, 2013; Segata et al., 2012) along with *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (Davis et al., 2013; Segata et al., 2012). *Bacteroides* and *Proteobacteria* are most present in human saliva (Keijser et al., 2008) with *Firmicutes* in high abundance in oral tissue and saliva (Davis et al., 2013). While each human individual has their own unique oral microbiome, a large percentage of the bacteria taxa in the mouth are consistent between individuals (Zaura et al., 2009). Healthy oral cavities contain *Streptococci*, *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus gordonii* and other related taxa. (Baker & Edlund, 2018). If there is disruption in the symbiotic relationship of healthy bacteria, disease can occur (Zaura et al., 2009). Saliva, as the first line of defense, reduces entrance and adhesion into mucosa (Marcotte, 1998). Oral diseases can occur from an imbalance of microbiota (Marcotte, 1998). Healthy individuals with no presence of periodontal disease still contained periodontal disease-causing bacteria, suggesting that these bacteria are part of the oral microbiome (Segata et al., 2012). A diet rich in non-carbohydrates enables healthy oral bacteria to flourish and keep the oral microbiome in symbiosis (Baker & Edlund, 2018). A diet rich in the consumption of carbohydrates decreases the diffusion of metabolites and results in an acidic oral environment from carbohydrate fermentation (Baker &

Edlund, 2018). Certain bacterial interactions in the oral cavity create a positive ecosystem to fight dental caries and fend off disease, while other bacterial interactions can cause dental diseases (Bowen, et al. 2018). Consumption of domesticated plants has a significant effect on the oral microbiome of humans (Bowen et al., 2018). The domestication of agriculture shifted the oral microbiota of humans, but the domestication of dogs by humans is another event that can help to better understand the relationships between humans and dogs and how the sharing of microbes may impact dog behavior and biology.

Dog Domestication:

Examining the oral composition of humans can help us to better understand the impact the oral microbiome of humans relates to dogs. To better analyze how the behaviors of humans affect the dog microbiome, domestication of dogs can further elaborate on the effects that humans had on dogs. While the exact date of the first domestication of dogs is widely argued due to different archaeological and molecular evidence, microsatellites and SNPs suggest the phylogeny of divergent lineages of modern dogs are of ancient origin and diverged from wolves (Larson et al., 2012). Archaeological data suggests that the domestication of dogs occurred around 33,000 to 10,000 years ago in Siberia, Israel, East Asia and the Middle East (Axelsson et al.,2013). It is theorized that the transition to a sedentary lifestyle by humans may have lead wolves near humans for access to food (Axelsson et al.,2013). Food sharing between dogs and humans impacted the digestive and metabolic system of dogs (Coelho, et al., 2018). Dogs have more copies of the amylase enzyme gene than wolves indicating a better ability to digest carbohydrates (Axelsson et al., 2013).

The role of domestication in dogs is not only seen biologically but also socially. While wolf puppies need exposure to humans prior to developing to understand human social cues, dog puppies do not and therefore this suggests that domestication has impacted dogs to be able to read human social cues (Wobber &Hare, 2009). Dog puppies are better at understanding human gestures than wolves even when wolves received more socialization and interaction with humans than the dog puppies did (Salomons et al. 2021). Cognitive changes can help to understand the impact of dog and human interactions in the past to better understand the effects of domestication in nonbiological ways. The evidence of cognitive and biological changes in dogs due to human manipulation of dog behavior could help to understand the oral microbial

composition of dogs and if there are similarities between human and dog oral ecosystem due to these factors.

Dog Oral Microbiome:

The canine oral microbiome consists of 99.5% bacteria and .01% archaeal and .46% eukaryotic SSU rRNA (McDonald et al., 2016). Dogs with a healthy oral microbiome have more aerobes in their oral microbiome (Davis et al., 2013). A previous 16S ribosomal RNA (rRNA) sequencing analysis study revealed that the most abundant taxa of bacteria in the dog oral microbiome is the phyla *Actinomyces*, along with *Granulicatella* and *Streptococcus* (Elliot et al., 2005). Dog oral bacterial phyla include Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Spirochaetes (Davis et al., 2013). There are one hundred sixty-two different taxa of Firmicutes found in the oral microbiome of dogs (Dewhirst et al., 2012). The bacteria found in healthy dog oral microbiome include *Moraxella zoohelcum, Neisseria shayeganii,* and *Pasteurellaceae*. Canine sex, size and age were not associated with significant differences of bacteria with principal component analysis (Davis et al.,2013).

Human and Dog Similarities:

Humans and dogs share 16.4% of the same plaque bacteria at the phyla and genus level (Ruparell et al., 2020). Bacteria from oral cavities of dogs reveal that at the phyla level, dogs share *Actinomyces, Porphyromonas, Fusobacterium, Neisseria* and *Streptococcus* with humans (Elliot et al., 2005). The location within the oral anatomy also affects the abundance of bacteria present for example, *Streptococcus* is low in dog plaque but more abundant in saliva (Elliot et al., 2005). *Neisseria shayeganii* was found in all four of the dog samples and their owners but not in the control groups (Oh et al. 2015). The identification of taxa at the species is harder to identify and distinguish similarities with humans (Elliot et al., 2005). The percentage of dog oral bacteria is low in humans (Oh, et al. 2015; Larson et al., 2012). 83.6% of the differences are seen at the species, genus and phylum level (Dewhirst et al. 2012). Differences can be attributed to the difficulty of canine oral bacteria to colonize human oral microbiome due to the basic pH of dog saliva that has to survive in the more acidic environment of human saliva (Oh, et al. 2015). In domesticated dogs, Gram-negative bacteria are associated with healthy dog oral microbiome, while in humans, it is associated with unhealthy oral plaque. Healthy human oral microbiomes are associated with Gram-positive bacteria, while interestingly, dogs with mild periodontitis have

higher gram-positive bacteria (Davis et al., 2013). Although humans and dogs share a small percentage of similar oral bacteria, *Granulicatella* species are closely related to *Streptococcus* and were at high frequency in dog saliva (Elliot et al.,2005). Similarities and differences of dog and human bacteria range within the phyla taxonomy and location in the oral cavity (Dewhirst et al, 2012; Ruparell et al.,2020; Elliot et al., 2005).

HYPOTHESES

Although dogs and humans have separate, distinguishable oral microbiomes (Dewhirst et al.,2012), could certain factors shift the bacterial composition and diversity of dog and human oral microbiomes? Short term visits to urban parks, gardens and natural environments reduce stress and improve both mental and physical health (Aerts et al., 2018). Increased exposure to outdoor environments has shown to change and increase skin and nasal microbial diversity (Selway et al., 2020). There is evidence of the benefits of frequently visiting outdoor environments and microbial changes occurring; therefore, would there be a change in the diversity of bacteria present oral microbiome of dogs who visit an external environment more?
The factor of age affecting the human oral microbiome has previously been studied in humans (Ferres et al., 2016; Song et al., 2013). For example, individuals under 35 had different proportions of oral bacteria than adults 36- 64 (Ferres et al. 2016). Children ages 3-18 showcased different bacteria present in their oral cavity during each developmental stage of dentition (Crielaard et al., 2011). Although these previous studies have shown that age affects the bacteria of the human oral microbiome, would this be seen in dogs?
Sex hormones are shown to affect the oral microbiome of humans (Lui et al., 2022). For example, female women who are pregnant showcase different bacteria in their oral microbiome during their trimesters along with differences to nonpregnant females (Fujiwara et al., 2017). Castrated laboratory mice have showcased transitioning to female like microbiome composition when their testosterone androgens were removed (Turkovetskiy et al., 2013). Sex differences are also seen; M-GWAS analysis reveals several loci associated with the tongue dorsum and salivary microbiome to be on the X chromosome (Lui, et al. 2022) Based on this current literature on the

effect of sex hormones on the oral microbiome, could these changes be seen in dogs? Could changes only occur due to sex hormones or secondary sexual characteristics? Co-inhabiting with other animals has been shown to affect the microbiome of individuals (Song et al., 2013). Adults' skin bacteria community is more similar to their own dog than other dogs (Song et al., 2013). Does cohabitation of other pets affect the oral composition of the dog sample?

Table 1 lists the hypotheses and predictions for these questions. These hypotheses will be tested to see if there is statistical significance of these factors and how the dog oral microbiome is affected. Analyses will be based on hypotheses from data accumulated from the Tudek Dog Park questionnaire, and dog samples and current literature will be used to compare to the human oral microbiome.

Table 1: Questions for Hypotheses with Predictions

Questions	Hypothesis	Prediction
Q1: Does the frequency of visiting an external environment affect the oral microbiome?	H (1): Visiting a dog park more frequently will increase the variety of bacteria present in dog oral microbiome	1: Dogs who visit the dog park more will have more diverse bacterial composition
Q2: Does age affect the composition of bacteria present in the oral microbiome?	H (2): The oral microbiome changes from juvenile to adult	1: Dogs under 1.5 of age will have different bacteria composition
Q3: Why are their sex differences in the composition of the oral microbiome?	H (3): There are sex differences due to sex hormones	N/A
	H (4): Due to primary sex differences	2: Will see no sex difference in dogs

Q4: Does cohabitation create similar bacterial composition?	H (5): The presence of cats in the household affects a dog's oral microbiome	1: Dogs living with cats will have similar composition to each other
	H (6): The presence of another dog in the household introduces new bacteria in the dog samples	2: There will be a difference in bacterial composition of solo dogs and dogs living with another dog

METHODS

Study Location

Saliva samples were collected from pet dogs in State College at the Tom Tudek Memorial Park in State College, PA (September-November 2022). The park is a fenced in park that allows dogs to be off leash. There is an area dedicated to small dogs and another area for medium to large dogs. Both areas of the Tudek Park have grass fields and access to water. The park is open to the public seven days a week during daylight. All procedures that were performed on animals were conducted in accordance with IACUC Dog Research Protocols and approved by Penn State IACUC protocol PROTO2021011946.

Data Collection

Dog owners were asked questions about their dogs. Certain questions used to analyze data about the oral microbiome included: sex of dog, spayed or neutered, juvenile or adult age, years of age in numeric digits, number of other dogs in household, if any, yes or no to cats in household, and attendance frequency of Tudek dog park. Owners gave permission to collect a saliva sample prior to collection. Saliva samples were taken with polymer cotton to retrieve saliva from lower cheeks and gums and under the gums for 1-3 minutes. The dogs did not eat a meal an hour prior to collection but a treat was held in front of their nose before collection to stimulate the production of saliva. Saliva samples were stored at -20 degrees Celsius until DNA

extraction. Sample ID names included A23, A33 A43, A54, A63, A73, A83, A93, B13, B23, B33, A43, B53, B63, B73, B83, B93, C13, C23, C43, C63, resulting in 21 dog saliva samples.

DNA Extraction

DNA was extracted from the dog saliva samples using the DNeasy Powersoil Pro Kit. Two separate extractions were conducted as per the manufacturer's instructions. Due to inadequate saliva content, 7 samples were unable to be used in the extraction therefore, so PCR was conducted on 14 dog saliva samples (A43, A54, A63, B13, B23, B33, B53, B63, B83, B93, C13, C23, C43, & C63) that were extracted by the DNeasy Powersoil Kit. Better saliva acquisition would ensure a larger sample size. Extraction blank controls (EBC) were performed to monitor microbial DNA present within the laboratory space.

PCR Amplification

PCR amplification was conducted on the 16S rRNA V-6 region using 515-F forward primer and 808-R reverse primer (Caporaso et al., 2012). CR was performed by Qubit BR dsDNA assay. A master mix was made by combining dH2O, 10x HiFi Buffer, MgSO4, dNTPs, F primer, HiFi polymerase. R primer or Extract was not added yet. One reaction was aliquoted into a set of labelled strip tubes (23 µL for all samples, 24 µL PCR negative). Controls and a PCR Negative were used to ensure proper gathering of saliva. 1 µL of R primer was added to each sample tube and mixed. Samples were briefly spun down using the strip tube adapter on microfuge. Tubes containing the samples were placed in PCR machine heat block, and the program was checked before starting the PCR amplification. Test strips were run through PCR nexus gradient for denaturation, annealing, elongation, and adenylation. Test tubes were removed after completion and stored at 4 degrees Celsius.

DNA Concentration

Qubit was used to quantify the DNA concentration of the PCR amplification by using the dsDNA DR Assay, lot number 2157155. There were 14 samples of dog saliva, 2 extraction blank controls and one PCR negative. Samples A4-C13 had a concentration above 45 (ng/µL). Samples C23, C43, C63 had a concentration under 15 (ng/µL). Extraction blank controls showed little contamination from PCR Amplification and were similar to the PCR Negative suggesting proper DNA extraction.

Purifying

Axy Prep was used for bead purification for 16S libraries. Samples were pooled in equal amounts. 1.1x AxyPrep beads were added, mix was flicked and spun down. Samples were incubated at room temperature for 15 minutes. Sample tubes were placed on magnetic rack for 15 more minutes. 800 µL of 80% EtOH was added to wash the beads. The 1.5 ml tubes were rotated 180 degrees in magnetic rack four times. Tubes were incubated for 1 minute and washed again. X30 µL of preheated EB buffer and Tween 0.05% was added to the tubes. The tubes were flick mixed and spun down and then incubated for 4 minutes. Tubes were placed on magnetic rack until beads formed a pellet and solution was clear.

Pooling

Libraries were pooled at equimolar concentrations after assessment on a QuBit. Dog saliva samples were submitted to the Huck Genomics Sequencing Core for a final quality assessment of the pooled samples and sequencing on an Illumina Miseq using a 2 x 150 bp kit.

Analysis

The sequence data was analyzed by the bioinformatic pipeline QIIME2. The raw sequence data was imported as a QIIME artefact. Pair end sequences were then demultiplexed. The quality plots showcased forward reads and reverse reads of the nucleotide sequences. The quality score of the reads was 36 at the highest. Data was truncated at 210 sequence bases because quality of nucleotide reads dropped after 210. The data was then cleaned for sequencing errors using DADA2. The nucleotide base reads were denoised for lower quality regions of reads. Denoising revealed data was high percentage of input passed filter 73-89%. After quality filtering the data, it was converted to a feature table with summaries of how many sequences associated with each sample and with each feature. Dog park meta data was used to tabulate sequences into representative sequences to create IDs to sequences. The variable of "park frequency" from Dog Park meta data used to analyze the alpha diversity. Beta diversity was used to find the dissimilarity in composition of bacteria in the variables: sex, age, cats in household and dogs in household. All commands used in QIIME2 to generate figures or run tests are in Table 2: QIIME2 Analysis Commands.

RESULTS

***Bacterial Composition*.** A stacked bar plot showing the bacterial composition of 14 dog saliva samples at the genus level from the 16S DNA sequencing is shown in Figure 1. The 30 most abundant bacteria present in the dog cohort are represented by different colors. The remaining bacteria counts are listed as "other". The most abundant bacteria, at the genus level, present in the dog sample are Proteobacteria Pasteurellaceae, Bacteroidota Porphyromonas, Proteobacteria Haemophilus, and Proteobacteria Conchiformibius. The bacterial composition of 14 dog saliva samples was also assessed at the species level (Figure 2). The 30 most abundant bacteria were graphed with the remaining bacteria labeled as "other" is similarly represented as Figure 1. The sampling depth for Figure 1 and Figure 2 was 44616 sequence counts for all of the bacteria phyla.

Alpha Diversity:

Park Frequency. To test the hypothesis H (1) of visiting a dog park more frequently will increase the variety of bacteria present in the dog oral microbiome, an alpha diversity analysis was analyzed on QIIME2 based on the frequency of dog park visits based on the questionnaire owners were asked about their dog. A qualitative measure of the samples' frequency at the dog park is represented as Faith's Phylogenetic diversity in Figure 3. Dog owners were asked how frequently they revisit the dog park. The possible choices to questions were "1-2 times per week", "3-6 times per week", "1-3 times per month", or "less than once per month". An alpha diversity box plot and shows the qualitative measure of community richness that incorporates phylogenetic relationship of the frequency of attending the dog park (Faith PD). The sample size of each answer was, (n=3) for "1-2 times per week", (n=5) for "3-6 times a week", (n=4) for "1-3 times per month", (n=2) for "less than once per month". The Faith PD score ranged from 6.0 to 11.0 of community richness similarity. We did not see significant differences in alpha diversity of the dogs who attended the park more or less frequently (Kruskal Wallis test; $H = 1.14$; $p = 0.77$). This suggests that visiting an external environment does not change diversity of bacteria present in the oral microbiome either if visiting occurs more or less frequently.

Beta Diversity: Juvenile and Adult. To test the hypothesis H (2) that the oral microbiome changes from juvenile to adult, a beta diversity analysis was conducted to assess the composition of the bacteria in the dog samples' saliva of juvenile and adult dogs. A PCoA plot showing the beta diversity (unweighted UniFrac and Bray Curtis distances) of distances to juvenile was examined (Figure 4 and Figure 5). Figure 4 shows the difference in oral bacterial composition of comparing the distances to adult with the adult dogs and comparing the distance of juvenile dogs with adult. Figure 4 shows two box plots with different means and upper and lower quartiles showcasing the dissimilarity of the two categories in their bacterial composition. The unweighted UniFrac Faith-pd distance is the qualitative measure of community dissimilarity that utilizes the phylogenetic relationships between the age groups. The ages of the 14 dogs in the sample ranged from .58 months to 5 years old, therefore the ages were categorized to "juvenile" age to dogs under 1.5 years and "adult" to older than 1.5 years. Sample size for "juvenile" was (n=6) and "adult" was (n=8). In Figure 5, juveniles are clustered closer together on the right side of the graph while adults are on the left side of the graph but are loosely clustered together. We did see significant differences in the composition (beta diversity) of dogs driven by their age (PERMANOVA; q value =0.02 from Adult and Juvenile; pseudo-F=1.90).

Female and Male Dogs: To test hypothesis H (4) that there are sex differences in the oral microbiome due to primary sex differences beta diversity was analyzed from male and female dogs from the sample cohort. Male sample count was (n=9) and female (n=5). A PCoA plot of unweighted UniFrac distances of Female and Male dogs is shown in Figure 6. Figure 6 shows dissimilarity of the bacterial composition of male and female dogs. Male and female dots in Figure 6 show no statistical significance in beta diversity. Graph 7 shows a plot of colored dots according to sex of dog. Male and female dogs show no dissimilarity in the plot because besides four male dogs clustered in the upper right side of the plot, male and female dogs are spread out in the graph near axis 2 and 3. The PERMANOVA results were a q- value of .16. The fitness of the test had a pseudo-F value of 1.24. This suggest that there is no impact on the bacteria composition of the dog oral microbiome due to primary sex differences. The hypothesis H (3) that there are sex differences due to sex hormones could not be analyzed due to only having 2 unfixed male dogs in the sample cohort and zero unfixed female dogs to compare to unfixed males. Although H (3) could not be predicted, Figure 12 displays the beta diversity of the two

unfixed dogs and fixed dogs. Although there are not enough samples in this category for analysis, the two unfixed dogs are close in proximity and on the right side of the graph indicate possible discussion.

Cats in Household: To test the hypothesis H (5) that the presence of cats in the household affects a dog's oral microbiome beta diversity was conducted on the dogs in the sample cohort that stated that they had a cat in the household from the questionnaire given to owners. Dog samples with a cat in the house was (n=4) and no was (n=10). A PCoA plot showing the beta diversity (unweighted UniFrac and Bray Curtis distances) of distances to No was examined (Figure 8 and Figure 9). Figure 8 shows the distance to Yes to a cat living in the household to the distance to No. The box plot shows the mean difference of having a cat is similar to no cat. The upper quartiles of No are greater than Yes and the lower quartiles of No cover more distance than Yes. Figure 9 shows no clustering of dissimilarity of colored dots for Yes to a cat compared to No to a cat. Figure 9 shows no clustering of dogs Yes to a cat in the household. We did not see significant differences in the composition (beta diversity) of dogs driven by the presence of a cat in the household. (PERMANOVA; p-value from Yes and No to Cat; pseudo-F=.8) This analysis suggests that cats do not influence a composition change in the dog oral microbiome even though they are different species.

Another Dog in Household: To test the hypothesis that the presence of another dog in the household introduces new bacteria in the dog samples, beta diversity was analyzed on the dogs from the sample cohort that live with another dog or one that is a single dog in the household.

A PCoA plot showing the beta diversity (unweighted UniFrac and Bray Curtis distances) of distances to No other dogswas examined (Figure 4 and Figure 5). A box plot shows the beta diversity of distances to 0 and 1 of other dogs in the household in Figure 10. Figure10 shows the dissimilarity of the presence of 1 or 0 other dogs in the household. The box plot shows a similar mean and upper and lower quartiles indicating there is no significant difference in the bacterial composition of the dog sample with 1 other dog and sample with no other dogs in the household. This is further represented in Figure 11, there is no clustering of dissimilarity of between the presence or absence of another dog in the household. We did not see significant differences in the composition (beta diversity) of dogs driven by their age (PERMANOVA; q value =0.89 from 0 and 1; pseudo-F=.68). Overall, this suggests that dogs who live with other dogs share a similar oral bacteria composition and do not introduce new bacteria in the oral microbiome.

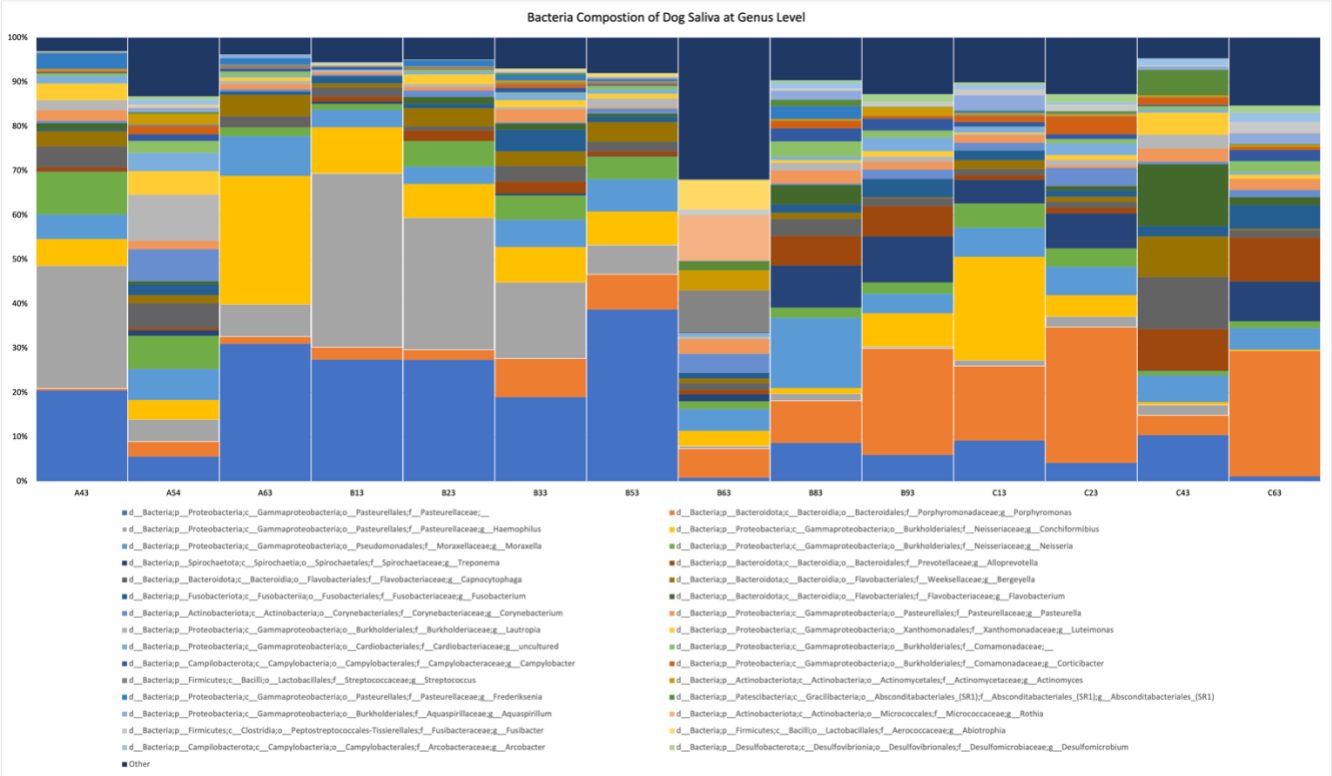

Figure 1: Bar plot showing the bacterial composition of 14 different dog samples at the genus level. The graph contains the 30 most abundant bacteria present in each dog saliva sample and the rest are listed as "other".

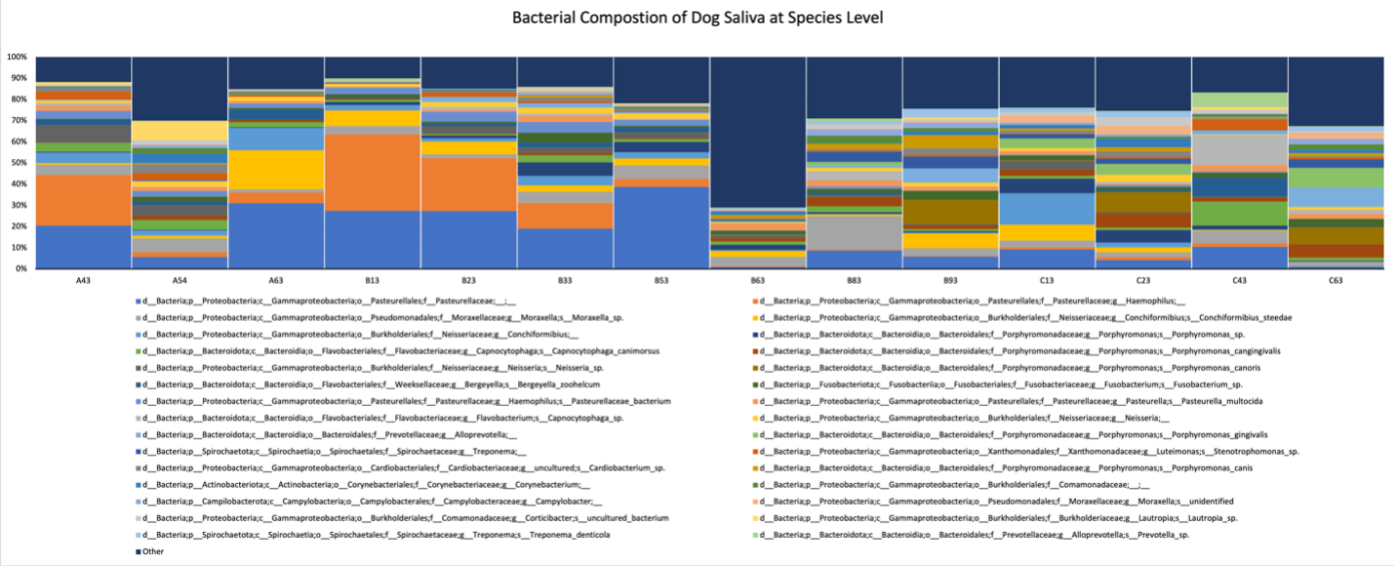

Figure 2: Bar plot showing the bacterial composition of 14 different dog samples at the species level for which sequences were obtained by QIIME2. The graph contains the 30 most abundant bacteria present in each dog saliva sample and the rest are listed as "other".

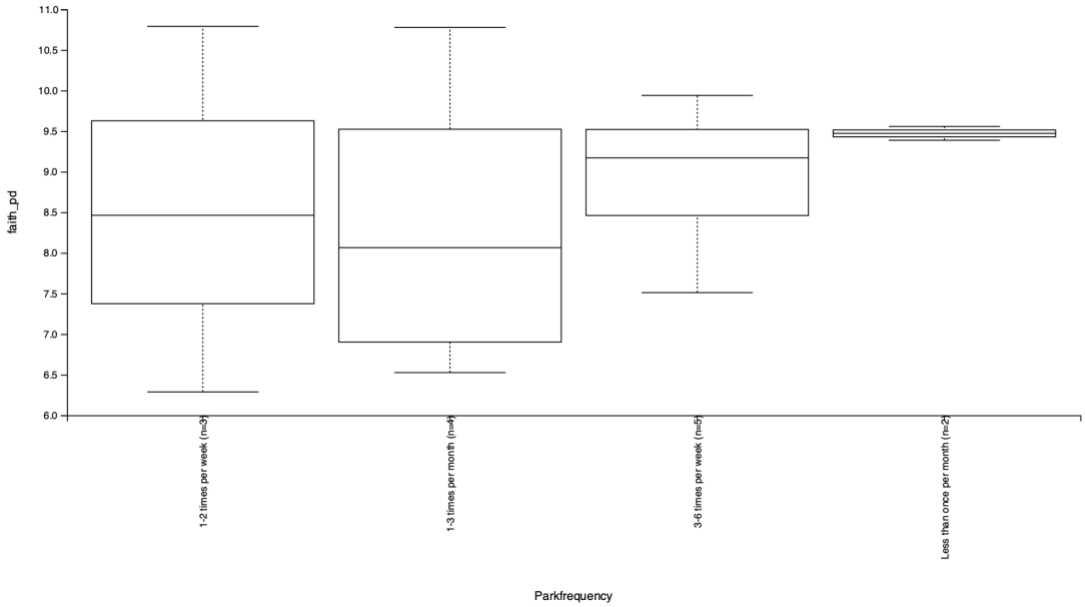

Figure 3: Faith-pd Boxplot of Park Frequency showing the alpha diversity of the bacterial diversity of samples who frequent the dog park compared to those who do not.

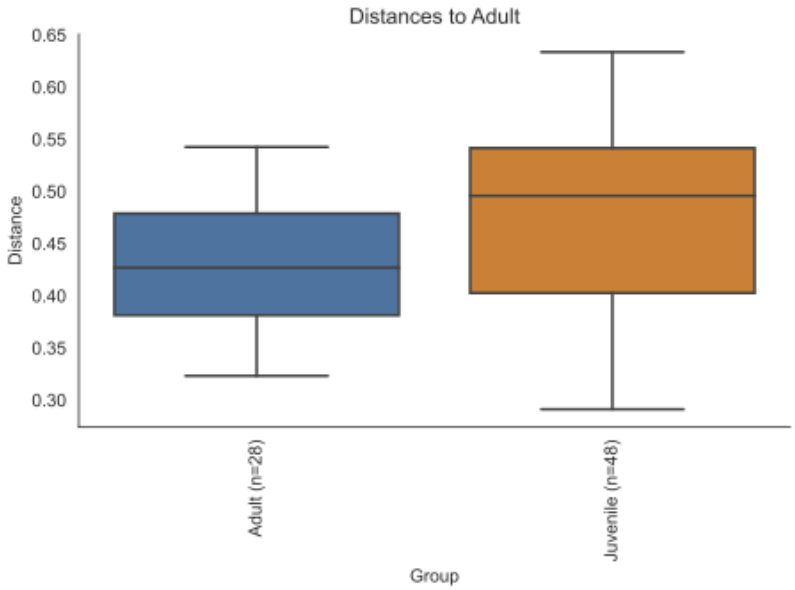

Figure 4: Box Plot of Distances to Juveniles

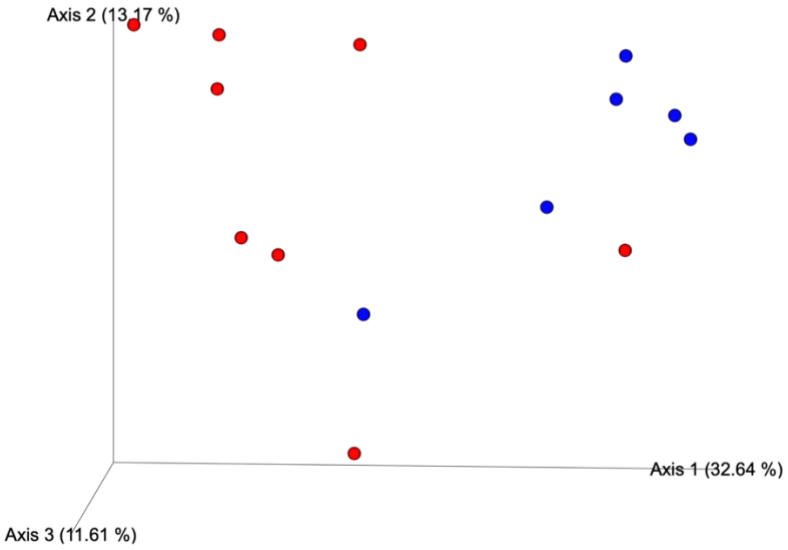

Figure 5: Bray Curtis Beta Diversity plot shows each sample as a dot according to their category, (juvenile in blue and adult in red).

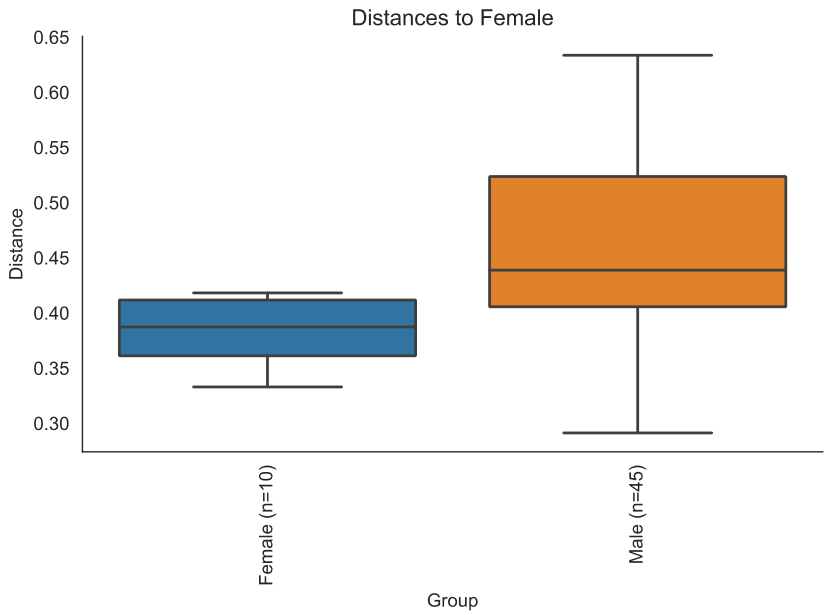

Figure 6: Bacterial Composition of Sex in Distances to Female Dogs

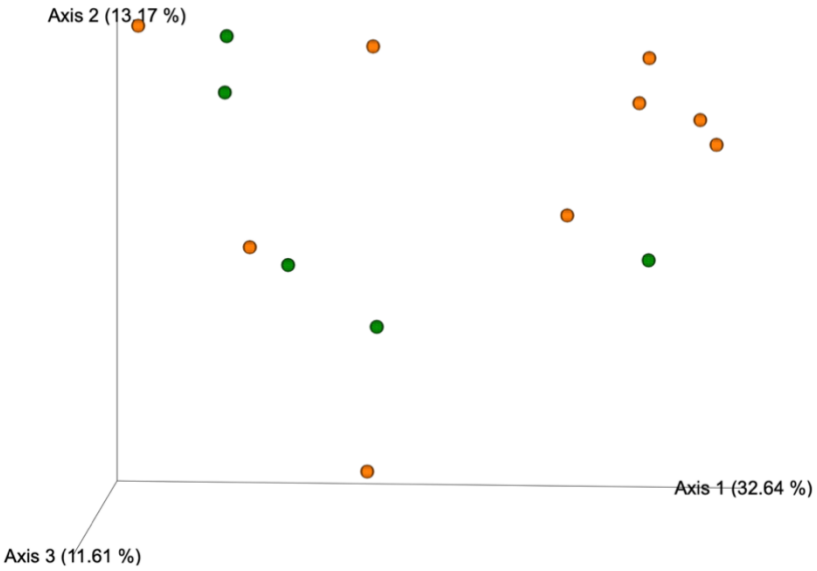

Figure 7: Bray Curtis Beta Diversity plot shows each sample as a dot according to their category, (male in orange and female in green).

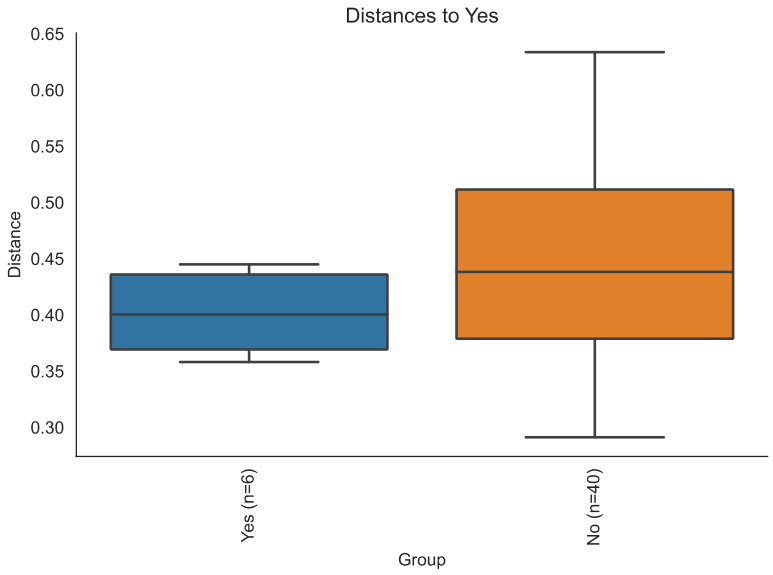

Figure 8: Composition of Cats in Household shows the dissimilarity to Yes to the presence of a cat living in the same house as a dog to yes and to no presence of a cat.

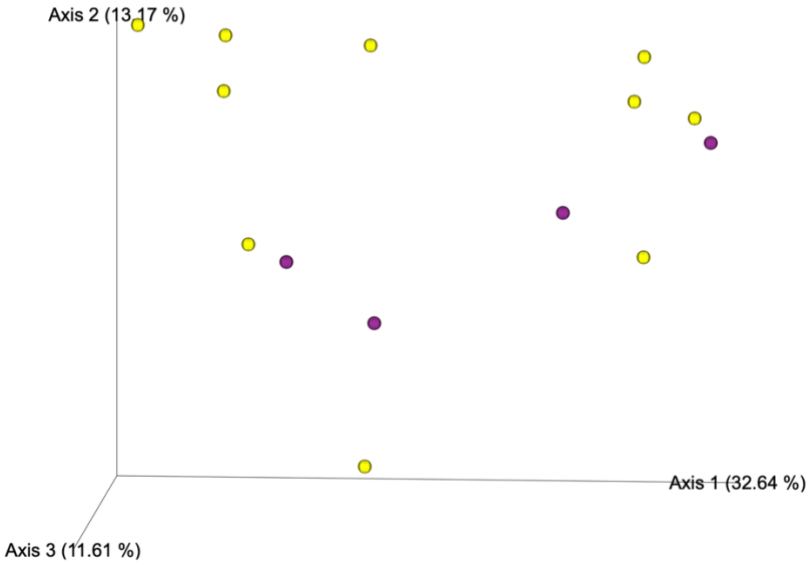

Figure 9: Bray Curtis Beta Diversity plot shows each sample as a dot according to their category, (Yes in purple and No in yellow).

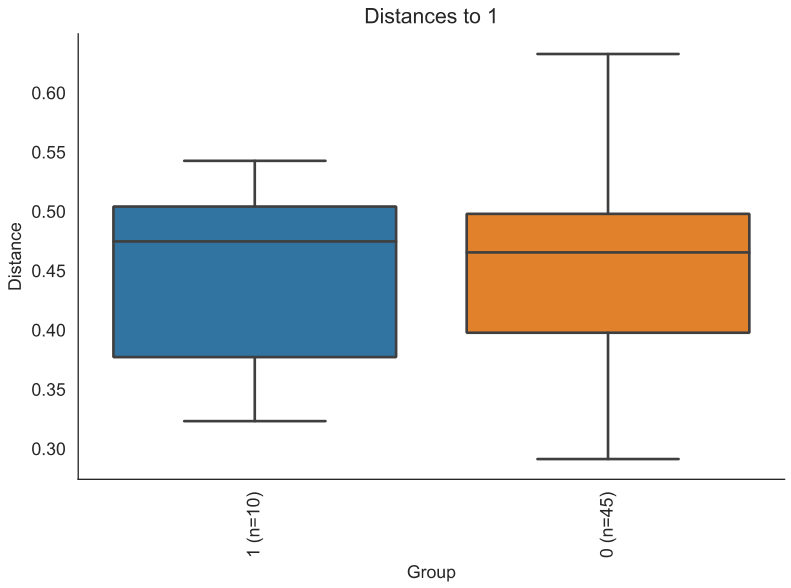

Figure 10: 1 or 0 Other Dogs in Household

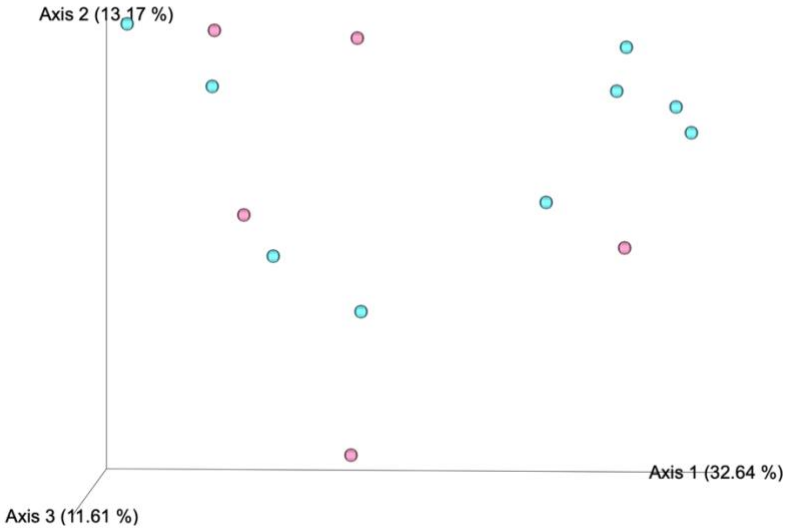

Figure 11: Bray Curtis Beta Diversity plot shows each sample as a dot according to their category, (1 in pink and 0 in blue).

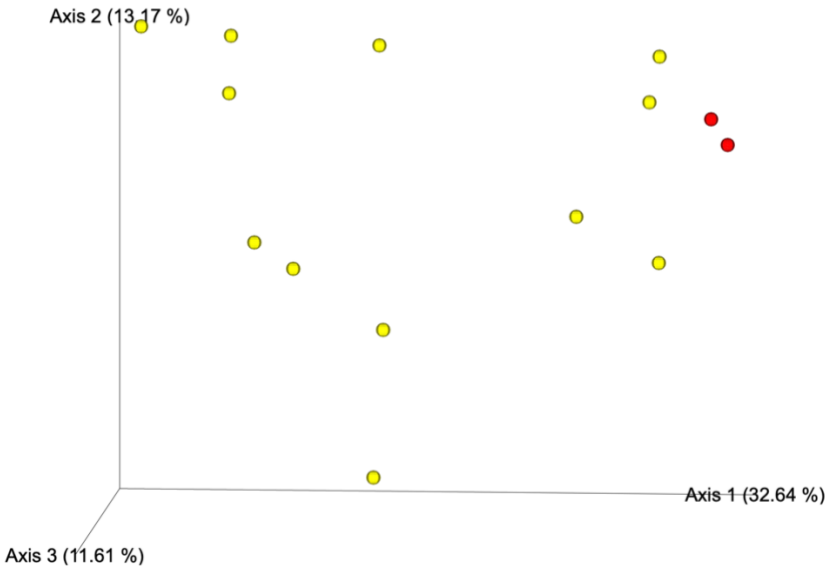

Figure 12: Bray Curtis Beta Diversity showing Unfixed (red) and Fixed (yellow) Dogs

DISCUSSION

Research needs to be improved in the understanding of the factors that cause changes to dog oral microbiome, especially if we want to better understand human and dog behaviors. In this study, the total bacterial composition of 14 dog samples who frequent Tudek Park was analyzed. Specific hypotheses based on park frequency, age, sex and other pet were introduced to better understand the impact of these factors on the sample cohort. The bacterial composition of dogs varied slightly among the dog sample indicating variety of possible oral bacteria present in dogs. Variation continued to occur based on the age of the dog. Certain factors, such as the age of dogs, showed statistical significance in beta diversity, while park frequency and other pets in the household showed statistical insignificance of bacterial composition. Based on results, sex of the dog also did not show significance but possible future considerations of having more intact dogs may result in a different analysis of this factor.

Bacterial Composition

The bacteria present in Figure 1 and 2 in the dog saliva samples include the phyla Proteobacteria, Spirochaetota, Fusobacteriota, Actinobacteria, Campilobacterota, Bacteroidota, Firmicutes, and Desulfobacterota. Besides Desulfobacterota, this composition has been seen by previous studies (Davis et al., 2013). Of these, Actinobacteria, Fusobacteria, Firmicutes, Bacteroides, and Proteobacteria are also found in the oral microbiome of humans (Davis et al., 2013; Elliot et al, 2005; Keijser et al.,2008; Segata et al., 2012). The most abundant bacteria, at the genus level, present in the dog sample are Proteobacteria Pasteurellaceae, Bacteroidota Porphyromonas, Proteobacteria Haemophilus, and Proteobacteria Conchiformibius. Although previous studies state that Streptococcus is one of the most abundant phyla in the dog oral microbiome (Elliot et al., 2005), this is not shared by this study. Figure 1 shows the percentage of genus of Streptococcus in the saliva of the sample cohort but, it is not in the top 20 bacteria genus present. Sample A43 and samples A63-B53 in Figure 1 have over 20% of their saliva composition contain *Proteobacteria pasteurellaceae*. These samples, A43, A63-B53 also contain a large percent of *Pasteurellaceae haemopilius* at the genus level. These bacteria are associated

with healthy oral microbiota in dogs (Davis et al., 2013). Bacteria represented by these two colors are of the same family but *pasteurellaceae* (blue) could not be identified to the specific genus level. A63 and C13 contain over 20% of *Neisseriaceae conchiformibius* (yellow). Although the genus is different, the species *Neisseria shayeganni* is present in humans and dogs (Oh et al., 2015; Davis et al., 2013). Samples B93, C13, C23 and C63 contain about 30% percent of *Porphyromonadaceae porphyromonas*, the genus Porphyromonas is present in the human oral microbiome as well (Elliot et al., 2005). While humans and dogs share the same genera of bacteria, when looking at the species level, there are differences. The identification of taxa at the species is harder to identify and distinguish similarities with humans (Elliot et al., 2005).

Dog Park Frequency

The H- (1) that visiting the dog park more frequently will increase the variety of the bacteria present in the dog oral microbiome had a prediction that dogs who visit the dog park more will have a more diverse bacterial composition. A resulting p-value of .77 for the alpha diversity of Figure 3 fails to reject the null hypothesis. The frequency of visiting the dog park shows no influence on the bacterial diversity of dogs who frequent the dog park compared to cohort that visits less. No differences in the richness of the bacteria in this variable is seen by frequency at the dog park. The Faith's phylogenetic diversity for categories “1-2 times per week” and “1-3 times per month” show a mean of similar diversity of their oral bacteria. The whiskers of highest and lowest values for these categories are similar to each other, even though the frequency of visiting the dog parks is very different. The sample size for “less than once per month” resulted in a skewed box plot variable due to a low sample count in that category (N=2). A larger sample size for this category may create a different box plot. The H value of 1.14 reveals that the test was fit. Although visiting the dog park more frequently had no significant difference on the oral microbiome diversity of the dog samples, visiting non-urban areas are beneficial in promoting a healthy microbiome (Haahtela, 2019). Along with benefits to the nasal and skin microbiomes (Selway et al.,2020). Despite a statistical insignificance of change in the oral microbiome, exposure to the outdoors has an impact on other parts of the body. Beyond the microbiome, walking or taking a dog to a park has also been shown to increase the physical activity of the owner (Veitch et al., 2019). The relationship of the dog park with the dog impacts both the owner

and the human further developing the behavior relationship of humans and dogs that has been occurring for thousands of years.

Juvenile and Adult Dogs

The H-(2) that the oral microbiome changes from juvenile to adult had a prediction that dogs under 1.5 years of age will have different bacteria composition than dogs older than 1.5 years. The beta diversity q-value of comparing the bacterial composition of juvenile and adult dogs was 0.02. The fitness of the data results was a pseudo-F value of 1.9. Based on these results, the null hypothesis is rejected. Based on the beta diversity, the oral microbiome changes from juvenile to adult, there is a statistical difference in bacterial composition of juvenile and adult dogs in the sample cohort.

Juvenile dogs show more dissimilarity with adult dogs than with other juveniles. Adult dogs show more bacterial composition dissimilarity with juvenile dogs but have more differences in their bacterial composition when compared to other adult dogs. Stages of human development show a similar difference of oral composition (Feres et al., 2016). Oral bacteria changes significantly from birth to three years of age (Song et al., 2013). Saliva from human children's deciduous teeth contained larger proportions of Firmicutes (*Streptococcus*) and Actinobacteria and smaller proportions of Bacteroidetes, Fusobacteria and Spirochaetes (Crielaard et al. 2011). Although some studies state that age is not a factor of change in the oral microbiome (Davis et al., 2013), the beta diversity test indicates a statistically significant difference of composition in juvenile and adult dogs.

Male and Female Dogs

H-(4) states that due to primary sex differences there is a difference in the oral microbiome of male and female dogs. A q-value of .16 for the beta diversity of the compositional dissimilarity signifies no statistical significance. This variable fails to reject the null hypothesis. These analysis results are similar to previous studies not finding a sex difference in the dog oral microbiome. No significant differences in sex of dog oral microbiome were detected (Isaiah et al.,2017). In humans, there are differences in the oral microbiome (Ma et al., 2019; Lui et al., 2022). Males had greater Firmicutes diversity than females in their saliva (Ma et al., 2019).

Females contained more *Streptococcus, Prevotella* and *Granulicatella* in their saliva compared to more *Campylobacter, Viellonella, Porphyromonas* and *Oribacterium* in males (Lui et al., 2022). Research on the human oral microbiome could be compared to dogs that there could be differences of sex based on factors that are not based on secondary sex characteristics. The prediction of H (4) was that there will not be a sex difference in dogs based on primary sex characteristics because of the state of the dogs in the sample being mostly fixed. H(3) could not be tested due to only have the sample have 2 male intact dogs, 12/14 dogs were fixed. Although hypothesis could not be predicted, graph 12 shows the beta diversity of fixed and unfixed dogs. The two red unfixed dogs are clustered close to each other on the right side of the graph, while the fixed dogs are mostly on the left side of the graph. This is interesting for the hypothesis H(3) that sex hormones could affect the oral microbiome. Sex hormones are relevant and contribute to oral bacterial difference in human males and females (Lui, et al. 2022). Hormones are further causal of oral bacteria change in human when looking at pregnant women. Pregnancy affects female oral microbiome, and the composition fluctuates during the trimesters (Fujiwara et al., 2017). A future study in nonfixed dogs may answer if hormones affect the dog's oral microbiome like it does in humans. Since hormones are an important factor of oral composition and diversity in humans, more intact dog samples would be interesting to test to see if a statistical significance can be reached with more unfixed dog samples. Then the impact of dog hormones can be assessed to see if there are similarities between humans and dogs based on hormones.

Presence of Other Pets in Household

H(5) states that the presence of cats in the household affects a dog's oral microbiome with a prediction off dogs living with cats will have similar composition to each other. A q-value of .73 for the beta diversity of the presence of a cat in the household fails to reject the null hypothesis. There is no significant dissimilarity in the presence of another cat creating a different composition of oral bacteria in dog sample. Cats have similar bacterial phyla as dogs (Dewhirst et al., 2015); therefore, no change in composition could be due to a similar bacteria present already. H(6) that the presence of another dog in the household introduces new bacteria in the dog samples with a prediction of there will be a difference in bacterial composition of solo dogs and dogs living with another dog. A q-value of .89 for the beta diversity of the presence of

another pet dog in the household fails to reject the null hypothesis. The presence of another dog does not affect the composition of the oral bacteria present in the dog sample. Cohabitation with another dog does not affect change in the oral microbiome based on these results but there could be a transfer of bacteria of different body parts. Humans with dogs as pets do show similar bacteria of skin phylotypes but not oral bacteria (Song et al., 2013). Therefore, analyzing bacteria on different body parts could reveal bacterial changes during cohabitation. Two unrelated people with dogs share more bacterial similarities of their skin phylotypes with each other than two cohabitating individuals with no dogs (Song et al., 2013). This could reveal that humans and dogs share similarities of bacteria on certain body parts due to cohabitation that could affect dogs as well.

CONCLUSIONS

Dogs and humans are not genetically similar but have an interesting share history. Studying the oral microbiome of dogs can help us to better understand human relationships and our effect on dogs. This study suggests that humans and dogs share similar oral bacteria at the genus level and that there are changes in the oral microbiome with age that are seen in dogs and from the current literature, is also seen in humans. This study has shown that age has an effect on the oral microbiome of dogs. Although null hypotheses failed to be rejected for park frequency, there is evidence from current literature that states the benefits of visiting a green environment, both biologically and socially. These benefit both humans and dogs, further developing human and dog relationship behaviors. While other pets in household and sex also failed to reject the null hypothesis, there is literature that supports the effect of hormones on the oral microbiome in humans and rats. Unfortunately, the study inly had two male unfixed dogs and the hypothesis of hormones affecting the oral microbiome of dogs could not be analyzed. Future considerations of this research topic include larger sample size to increase statistical significance of analyses. Unfortunately, sample collection frequency slowed down due to colder weather and less dogs and owners frequenting the dog park. Better acquisition of dog saliva from sample collection may have ensured more samples to be extracted and analyzed. Ensuring a sample size that has

fixed and unfixed dogs would enable researchers to better understand the effects of hormones on the oral microbiome. Based on the factors that the null hypothesis failed to be rejected it would be interesting to see if there are bacterial changes on other parts of the dog's body and not just the oral cavity that could show a similarity of bacterium in dogs and humans in order to better understand the behaviors of humans and dogs and this relationship effect on their biology.

SUPPLEMENTAL INFORMATION

Table 2: QIIME2 Analysis Commands

Steps Chronology	Command
Importing Data	qiime tools import \ --type 'SampleData[PairedEndSequencesWithQuality]' \ --input-path raw_fastq\ --input-format CasavaOneEightSingleLanePerSampleDirFmt \ --output-path demux-paired-end.qza
Check UUID	Qiime tools peek demux-paired-end.qza
Summary of Sequence Quality	qiime demux summarize \ --i-data demux-paired-end.qza \ --o-visualization demux-paired-end.qzv
Denoise DADA2	qiime dada2 denoise-paired \ --i-demultiplexed-seqs Data_Import_and_Quality/demux-paired-end.qza \ --p-trunc-len-f 210 \ --p-trunc-len-r 210 \ --o-representative-sequences Data_Import_and_Quality/representative-sequences.qza \ --o-table Data_Import_and_Quality/table.qza --o-denoising-stats Data_Import_and_Quality/denoising-stats.qza
	qiime metadata tabulate \ --m-input-file Data_Import_and_Quality/denoising-stats.qza \ --o-visualization Data_Import_and_Quality/stats-dada2.qzv
Feature Table and Data Summaries	qiime feature-table summarize \ --i-table Data_Import_and_Quality/table.qza \ --o-visualization Data_Import_and_Quality/table.qzv \ --m-sample-metadata-file Data_Import_and_Quality/DogParkMetadata.tsv qiime feature-table tabulate-seqs \ --i-data Data_Import_and_Quality/representative-sequences.qza \ --o-visualization Data_Import_and_Quality/rep-seqs.qzv
Filter out Singletons and Controls	qiime feature-table filter-features \ --i-table Data_Import_and_Quality/table.qza\ --p-min-frequency 2 \ --o-filtered-table Data_Import_and_Quality/Dog_NoSing.qza
Double Check Feature Table	qiime feature-table filter-samples \ --i-table Data_Import_and_Quality/Dog_NoSing.qza \ --m-metadata-file Data_Import_and_Quality/DogParkMetadata.tsv\ --p-where "YNSample='sample'" \ --o-filtered-table Data_Import_and_Quality/Dog_BioSamplesOnly.qza qiime feature-table summarize \ --i-table Data_Import_and_Quality/Dog_BioSamplesOnly.qza \

	--o-visualization Data_Import_and_Quality/Dog_BioSamplesOnly.qzv
Taxonomic Analysis	qiime feature-classifier classify-sklearn \ --i-classifier silva-138-99-515-806-nb-classifier.qza \ --i-reads representative-sequences.qza \ --o-classification taxonomy.qza qiime metadata tabulate \ --m-input-file taxonomy.qza \ --o-visualization taxonomy.qzv
Differential Abundance Test	qiime feature-table filter-samples \ --i-table table.qza \ --m-metadata-file DogParkMetadata.tsv \ --p-where "[Sex]='Female'" \ --o-filtered-table Female-table.qza
Phylogenetic Taxonomy	qiime diversity core-metrics-phylogenetic \ --i-phylogeny rooted-tree.qza \ --i-table table.qza \ --p-sampling-depth 1103 \ --m-metadata-file DogParkMetadata.tsv \ --output-dir core-metrics-results
Genus Level	qiime taxa collapse \ --i-table table.qza \ --p-level 6 \ --o-collapsed-table table-L6.qza \ --i-taxonomy taxonomy.qza qiime feature-table summarize \ --i-table table-L6.qza \ --m-sample-metadata-file DogParkMetadata.tsv \ --o-visualization table-L6.qzv
Species Level	qiime taxa collapse \ --i-table table.qza \ --p-level 7 \ --o-collapsed-table table-L7.qza \ --i-taxonomy taxonomy.qza qiime feature-table summarize \ --i-table table-L7.qza \ --m-sample-metadata-file DogParkMetadata.tsv \ --o-visualization table-L7.qzv
Rarefy L6 Table	qiime tools export \ --input-path table-L6.qza \ --output-path exported-table-L6-feature-table.tsv

	qiime feature-table summarize \
	  --i-table table-L6_rare44616.qza \
	  --o-visualization table-L6_rare44616.qzv

Rarefy L7 Table	qiime feature-table rarefy \
	--i-table table-L7.qza \
	--p-sampling-depth 44616 \
	--o-rarefied-table table-L7_rare44616.qza
	
	qiime feature-table summarize \
	  --i-table table-L7_rare44616.qza \
	  --o-visualization table-L7_rare44616.qzv

Export L6 to txt.	qiime tools export \
	  --input-path table-L6_rare44616.qza\
	  --output-path table-L6_rare44616
	
	biom convert \
	--input-fp table-L6_rare44616/feature-table.biom \
	-o table-L6_rare44616/table-L6_rare44616.txt \
	--to-tsv \
	--table-type 'OTU table'

Export L7 to txt.	qiime tools export \
	  --input-path table-L7_rare44616.qza\
	  --output-path table-L7_rare44616
	
	biom convert \
	--input-fp table-L7_rare44616/feature-table.biom \
	-o table-L7_rare44616/table-L7_rare44616.txt \
	--to-tsv \
	--table-type 'OTU table'

Alpha and Beta Diversity	qiime diversity core-metrics-phylogenetic \
	  --i-phylogeny rooted-tree.qza \
	  --i-table table.qza \
	  --p-sampling-depth 44616 \
	  --m-metadata-file DogParkMetadata.tsv \
	  --output-dir core-metrics-Alpha-Beta-results

Alpha Diversity “Park Frequency” from dog park meta data	qiime diversity alpha-group-significance \
	  --i-alpha-diversity core-metrics-results/faith_pd_vector.qza \
	  --m-metadata-file DogParkMetadata.tsv \
	  --o-visualization faith-pd-group-significance.qzv
	
	qiime diversity alpha-group-significance \
	  --i-alpha-diversity core-metrics-results/evenness_vector.qza \
	  --m-metadata-file DogParkMetadata.tsv \
	  --o-visualization evenness-group-significance.qzv

PERMANOVA for Beta Diversity of "SEX" from dog park meta data	qiime diversity beta-group-significance \ --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \ --m-metadata-file DogParkMetadata.tsv \ --m-metadata-column Sex \ --p-pairwise \ --o-visualization unweighted-unifrac-Sex-group-significance.qzv
PERMANOVA for Beta Diversity of "JuvenileAdult" from dog park meta data	qiime diversity beta-group-significance \ --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \ --m-metadata-file DogParkMetadata.tsv \ --m-metadata-column JuvenileAdult \ --p-pairwise \ --o-visualization unweighted-unifrac-JuvenileAdult-group-significance.qzv
PERMANOVA for beta diversity of "Other Dogs" from dog park meta data	qiime diversity beta-group-significance \ --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \ --m-metadata-file DogParkMetadata.tsv \ --m-metadata-column OtherDogs \ --p-pairwise \ --o-visualization unweighted-unifrac-OtherDogs-group-significance.qzv
PERMANOVA for beta diversity of "CatsYN" from dog park meta data	qiime diversity beta-group-significance \ --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \ --m-metadata-file DogParkMetadata.tsv \ --m-metadata-column CatsYN \ --p-pairwise \ --o-visualization unweighted-unifrac-CatsYN-group-significance.qzv
PERMANOVA for beta diversity of "Fixed"	qiime diversity beta-group-significance \ --i-distance-matrix diversity-core-metrics/unweighted_unifrac_distance_matrix.qza \ --m-metadata-file DogParkMetadata.tsv \ --m-metadata-column Fixed \ --p-pairwise \ --o-visualization unweighted-unifrac-Fixed-group-significance.qzv

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ACADEMIC VITA

Lauren Marina Stroupe

Lms7001@psu.edu
www.linkedin.com/in/lauren-stroupe-9962a4201

Education

The Pennsylvania State University, University Park, PA Expected May 2023
College of the Liberal Arts at Penn State
Bachelor of Science in Anthropological Science, Biological Anthropology Option, Minor: Art History
International Studies Institute (ISI), Florence, Italy Feb.-May 2022

Honors

Paterno Fellows Program Fall 2019-Present
Schreyer Honors College Fall 2020- Present

Experience

Teaching Assistant, Class: Cultural Diversity Fall 2021

- Held responsibility of managing grading of 120 students' assignments.
- Assisted Dr. Doug Bird in creating study guides and study sessions for exams
- Held weekly office hours to answer questions about lecture topics

Research in Micro Archaeology Lab Fall 2022-Present

- Conducting primary research with Dr. Laura Weyrich on how humans affect the oral microbiome of dogs
- Collected oral microbiome samples of dogs and collected systematic data on dog behavior and cognition
- Performed DNA extraction from canine samples using DNeasy Powersoil Kit and PCR

Additional Experience

Phyrst Inc. Central Reservation Restaurant, State College, PA, Server Jan. 2021- Present

- Responsible for training new employees with their duties within the restaurant
- Provided hospitality to 100s of guests at a fine dining establishment to ensure a positive experience
- Maintaining a clean environment during the Covid-19 pandemic and making sure proper sanitation protocols were followed by guests and staff