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Reduced sleep quality is a common problem in the US, linked to increased risk for several disease conditions. Current evidence indicates an association between the gut microbiome and sleep modulation. With the ease of access to the oral cavity for examination and sampling, correlations between the gut and oral microbiome have clinical implications for developing health screening tools. Based on this relationship, this study examined whether changes in the gut microbiome of chronically sleep-restricted subjects were reflected in their oral microbiome. Mice models were placed on chronic sleep restriction for six weeks via the Modified Multiple Platform Method. Additionally, LPS injections were given to mice four hours prior to euthanasia (saline given to control group). Oral swabs and fecal samples were collected to profile the microbiomes, characterized using Miseq for sequencing the V4 region of the 16S rRNA gene.

The oral samples exhibited less diverse range of phyla, favoring Proteobacteria and Firmicutes; while the gut samples exhibited more diverse range. Among the oral microbiome profile, only Firmicutes indicated a possible association to sleep restriction with marked decrease in abundance compared to the control mice's oral samples. Among the fecal microbiome phylum analysis, two samples (S13, S21) consistently clustered away from the rest were under the conditions of no sleep restriction with LPS injection. Unexpectedly, not all of the mice which received LPS injections clustered together on PCoA. Additional studies with larger sample size are necessary to further understand the complex mechanisms taking place.

ORAL AND GUT MICROBIOME

IN CHRONIC SLEEP RESTRICTION MICE MODELS

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ORAL AND GUT MICROBIOME

IN CHRONIC SLEEP RESTRICTION MICE MODELS

INTERNSHIP PRACTICUM REPORT

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INTRODUCTION

The human body houses a significant number of microorganisms, which can include viruses, bacteria, fungi, and protozoa [1]. These microscopic communities comprise the human microbiome and exhibit a highly complex relationship of host and occupant. Microbes that live symbiotically or commensally with their human hosts, without causing disease, are known as normal microflora. Conversely, parasitic microbes cause harm to humans. It becomes further complexed when normal microflora become pathogenic to their hosts, deviating from the established bacteria-host dynamic. Thus, microbiome research is crucial in better understanding the specific relationships that exists between humans and our microscopic inhabitants that can play a role in determining our state of health or disease.

The NIH Human Microbiome Project (HMP) consortium sampled various sites of the body in a large cohort (*n=242*) in efforts to comprehensively profile the human microbiota. Among eighteen anatomical sites in females and fifteen anatomical sites in males sampled, the oral and gut compartments were indicated to be two of the most microbially diverse sites in the human body [2][3].

Studies of gut microbiome are increasingly revealing its link to major health conditions, such as: neurodegenerative diseases [4], diabetes [5], autism [6], obesity [7], and IBD [4, 5, 6, 7]. Additionally, the gut microbiome has been shown to have roles in a number of physiological processes. An experiment transplanting microbiome from obese Ig-A deficient mice to germ-free mice resulted in conference of insulin resistance and inflammation that the germ-free mice did not previously have. Thus, researchers considered that host metabolic changes could be directed by

constituents of the gut microbiome [7]. While microflorae are able to affect its host, host organisms are also able to exert modulatory action onto their microbiome constituents. A study investigating the relationship between gut microbiota and human hosts' circadian rhythm concluded that the host's endogenous melatonin had regulatory roles on gut microbiota activity. Swarming behavior (forming colonies in concentric rings) and motility in *E. aerogenes* cultures were used as indicators of activity coinciding with circadian rhythm. *E. aerogenes* cultures given melatonin expressed markedly increased swarming activity compared to melatonin-deficient cultures. This same study also found that cultures given melatonin had synchronized circadian phases versus more varied phases between the replicates of the melatonin-deficient cultures [8]. However, another study suggests it is the microorganisms in the gut microbiome that have the ability to alter host hormonal regulation and affect host circadian rhythms [9]. Due to the complex nature of the human body and its microbial communities, as well as the numerous other internal and external factors that influence the state of an individual's health, it remains unclear whether the microbiome is the key player affecting change or a downstream result of other effectors.

The oral microbiome's interconnection to major disease conditions are beginning to be elucidated as well, making it an emerging topic of interest in discussions of human health. Poor oral hygiene allows opportunity for increased oral bacterial load. The eventual development of dental caries and deterioration of gingival health can escalate to cause a number of oral diseases, such as cavitation, dentition loss, gingivitis, and periodontal disease [10]. While the oral microbiota's role in oral health has been well established, it's role in systemic disease is still being explored. For example, the state of the oral microbiome has been linked to dementia. An early suggestion of poor oral hygiene habits had a correlation with dementia from studies in the 1950s of the Swedish Twin Registry. Premature tooth loss was used as an indirect indicator of poor oral

hygiene. They found that premature tooth loss, occurring before the age of thirty-five years old, was found to be statistically significant in identical twins who developed dementia [11]. A later eighteen-year, longitudinal cohort study used teeth brushing habits as a direct indicator of oral hygiene. There was a 22-65% increased risk of dementia associated with poor oral hygiene [12]. Cardiovascular disease has also been linked to oral microbiota activity [13]. Studies have found constituents of oral bacteria, such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, in carotid atheromas, although the causal relationship still remains unclear [13][14]. Nevertheless, it is becoming more evident that the oral microbiome's activity can have effects beyond the confines of the oral cavity structure.

The direct structural connection between the oral cavity and gut allows for constant interaction among their microbial communities. As such, it is important to understand the type of relationship that exists between the two environments. A metagenomic study analyzing the oral and gut microbiomes found 23,961,508 microbial genes in the mouth and 22,254,436 microbial genes in the gut. There was an overlap of 549,610 microbial genes between the two sites, meaning only 2.29% of oral microbes can be found in the lower gastrointestinal tract [15]. The low percentage may be due to the inability of most oral bacteria to colonize the healthy gut [16]. However, in several disease states, "oral bacteria-mediated gut dysbiosis" was observed, meaning oral microbes that would otherwise not be present in the gut were able to establish residence at intestinal sites [17]. A study involving colorectal cancer found that more than 40% of the patients in their sample had identical strains of *F. nucleatum*, a commensal oral bacterium, in both their saliva and the colorectal cancer site [18].

With the ease of access to the oral cavity for examination and sampling, correlations between the gut and oral microbiome have clinical implications for the development of screening

tools for health conditions. The prominence of sleep problems and the association of increased inflammation with poor sleep quality [19] makes it a greatly relevant health issue of interest for the background of this microbiome study.

Reduced sleep quality is a widespread problem in the US. In 2014, the CDC found that 35.2% of all adults in the United States had insufficient sleep duration (defined as less than seven hours) [20]. In another population study, 29.1% self-rated to have poor sleep quality with characteristics of difficulty initiating sleep, problems maintaining sleep, and still feeling fatigue upon waking up [21]. Inadequate sleep is linked to increased risk for several conditions, such as obesity, cardiovascular disease, type 2 diabetes, and Alzheimer's disease [22]. Chronic sleep deprivation can result in the activation of inflammatory responses. Numerous inflammatory mediators are noted to be elevated with sleep deprivation, such as monocytes, neutrophils, and several interleukin types [23].

This project compared profiles of mice oral and gut microbiomes to identify overlapping bacterial taxa characteristic of stress reaction in chronic sleep deprivation.

BACKGROUND

This study will be observing the following phyla in the composition of microbial communities of the oral and gut sites in murine models: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria.

Firmicutes

Firmicutes are the most abundant bacterial phyla in the lower gastrointestinal microbiome. Profiling of the cecal microbiota of obese and lean mice using Sanger sequencing found that 60-80% of the sequences belonged to the Firmicutes phylum [24]. While not the most abundant bacterial phylum in the mouth, Firmicutes also comprise a large proportion of the oral microbiome [25]. Firmicutes aid in the breakdown of complex carbohydrates through fermentation. A study of obese mice missing the leptin gene showed a significant increase of Firmicutes (and decrease of Bacteroidetes) with an associated increase in the microbial production of enzymes involved in the breakdown of complex carbohydrates and short chain fatty acids. This positive association is thought to be caused by Firmicutes' ability to process carbohydrates in the intestinal tract that would otherwise be unabsorbed, thus increasing energy recovery and contributing to obesity [26]. Increased abundance of Firmicutes have also been seen in IBS patients [27]. Not only does an overpopulation of Firmicutes signify health complications, but so does a significant decrease of intestinal Firmicutes. While it is still unclear whether Firmicutes' contribute causing disequilibrium or is rather an effect of dysfunction, its diminished capacity is linked to major

depressive disorder [25], Alzheimer's disease [26], colorectal cancer [28], and type II diabetes [28, 29, 30, 31].

Bacteroidetes

In humans, Bacteroidetes comprise a substantial part of the gut microbiome and is the most abundant bacterial phylum found in the oral microbiome [32]. The *Bacteroides* genus, categorized under the Bacteroidetes phylum, are the predominant anaerobic bacteria occupying the human intestinal environment [33]. Bacteroidetes, specifically the *Bacteroides* genus in the gut, have roles in carbohydrate metabolism through the expression of essential enzymes, production of short chain fatty acid (i.e. propionate, butyrate), and pathogenicity when under opportunistic circumstances [34]. Obesity and the association of decreased Bacteroidetes, especially in the context of the Firmicutes-to-Bacteroidetes ratio, have been well documented in microbiome research [35]. Additionally, studies on mice models fed a high fat "Western" diet have also resulted in significant loss of Bacteroidetes abundance [36]. In contrast, studies of type II diabetes models exhibited increase in Bacteroidetes proportions [37]. In the oral cavity, Bacteroidetes remained the most abundant phylum and even increased 26% in patients with periodontitis as compared to healthy patients [32].

Actinobacteria

Among the four major phyla discussed, Actinobacteria and Proteobacteria together account for roughly 10% of the gut microbiome [38]. Actinobacteria are a group of Gram-positive bacteria. Although it is a minority population of the gut, Actinobacteria have crucial roles in nutrition, chemical detoxification, lipid metabolism, gut maintenance, and host health [39]. Characteristic of

many normal intestinal microflora, Actinobacteria also produce products through conversion and fermentation that are able to be used by the host. Thus, it's dysbiosis has been linked to several health complications. A mouse study investigating genetically lean mice, obese mice, and lean mice fed a high-fat "Western" diet indicated elevated Actinobacteria proportions in both the obese group and the high-fat diet group [24]. Another study comparing lean and obese humans also indicated increased gut Actinobacteria in the obese group [29]. However, a decrease in the *Bifidobacteria* genus, belonging to the Actinobacteria phylum, affected the maintenance of the protective intestinal barrier. As a result, the increased intestinal permeability can give opportunity for increased lipopolysaccharide (LPS) absorption and lead to the triggering of chronic inflammatory responses [38]. The effects of Actinobacteria are not limited to the gastrointestinal system. An examination of patients with mood disorders showed elevated gut Actinobacteria in patients with major depressive disorder as well as bipolar disorder [40]. In the oral cavity, Actinobacteria were found to be greatly diminished in patients with periodontitis as compared to healthy patients [32].

Proteobacteria

Proteobacteria are a minor bacterial phylum in the intestinal microbial community. The dysbiosis of proteobacteria, mainly in the direction of increased intestinal abundance, has been identified with type II diabetes, obesity, and post gastric bypass patients [41]. Proteobacteria also have shown involvement with cardiovascular pathology. For example, high levels of Proteobacteria have been found in atherosclerotic plaques. Thus, it is theorized to have pro-inflammatory and plaque activating properties [42]. While Proteobacteria have low abundance in

the gut, it is much more prominent in the oral cavity with average abundance 11% and highest abundance of 17-37% [44][41].

METHOD OF PROCEDURE

Texas Christian University's (TCU) Neurobiology of Aging graduate labs were an immensely helpful resource to the development of this project. Their various ongoing studies and sample collections provided an opportunity for collaboration that greatly addressed the time limitations of this project. The main study at TCU was investigating central inflammation in chronic sleep restriction stress models. To prevent the introduction of any unwanted variables or unknowns to TCU's active study, sample collection for this project was taken from the subjects, post-mortem.

The mice used were C57BL/6 male mice stock obtained from the Jackson Laboratory and bred in the TCU vivarium. Test mice were placed on a chronic sleep restriction schedule for six weeks, from 8am to 6pm (ten hours) daily, via the Modified Multiple Platform Methods (MMPM). Mice were placed in home groups of 3-4 into rat boxes with fourteen small platforms (roughly 3 cm in diameter and 3 cm in height) placed in a staggered pattern (Figure 1). Water filled the bottom of the boxes up to 1 cm below the surface of the platforms. When mice reached REM sleep and lost muscle tone, they fell off of the platforms into the water and were suddenly awoken. Mice in the control condition were transferred to new, clean cages identical to their home cages at 8am and transferred back to their home cages at 6pm to control for the handling of animals twice a day. Specimens remained in their social groups of 3-4 for the entire experimental protocol. TCU had multiple batches of mice in their study. Oral samples for the oral microbiome project addressed in this paper were collected specifically from TCU's batch 5 containing forty-eight mice.

The mice also received one intraperitoneal injection of either lipopolysaccharide (LPS) or saline (control) four hours before they were anesthetized for brain tissue collection. This procedure was conducted the morning after the last sleep restriction day. Thus, they were not exposed to the sleep restriction boxes that day. LPS is a surface glycolipid produced by Gram-negative bacteria which creates the permeability barrier to protect itself and also expresses bacterial pathogenicity [45]. Cytokine expression due to the LPS peaks four hours after intraperitoneal injection. Thus, the LPS procedure was a part of TCU's protocol for examining central inflammation in the brain.

Figure 1: Modified Multiple Platform Methods (MMPM) for stress model using sleep deprivation. (image credit: TCU Neurobiology of Aging graduate labs, Kelly Brice)

Sample Collection

TCU labs collected fecal samples throughout their chronic sleep restriction study. It was possible to use fecal samples opportunistically collected in the sleep deprivation study for comparison with oral samples.

Viable oral swab samples were collected from seven mice (n=7), with end-point fecal specimens from the same seven mice retrieved from the TCU labs' sample collections (Table 1). The sample IDs include designations of 'm' for mouth or 'f' for fecal origin. Additionally, mice

were placed in the four possible conditions: sleep restricted with saline injection (s/s), sleep restricted with LPS injection (s/L), control home cage with LPS injection (c/L), or control home cage with saline injection (c/s).

Table 1: Samples and Conditions in the study Group designations: (s/s) sleep restricted, saline injection (s/L) sleep restricted, LPS injection (c/L) control, LPS injection (c/s) control, saline injection

Group	**Sample ID**	**Condition: Sleep restricted or control home cage**	**Injection Treatment: LPS vs Saline (control)**
A (s/s)	R24	sleep restricted	saline
	S12	sleep restricted	saline
B (s/L)	S13	sleep restricted	LPS
	R21	sleep restricted	LPS
C (c/L)	S23	control	LPS
	S31	control	LPS
D (c/s)	R34	control	saline

Oral swab samples were collected using Puritan Sterile Polyester Tipped Applicators. The mice were each swabbed for thirty seconds, in the following order of oral regions: lingual, both buccal areas, palate, and gingiva. Swabs were then placed into autoclaved 1.5 ml Safe-Lock tubes with the plastic swab handle trimmed down to allow for closure of the tubes. The samples were stored at -20°C until DNA extraction.

DNA Extraction and Clean-Up

The MP Bio's Fast DNA SPIN Kit for Soil was used for DNA extraction. Due to over-dilution of my DNA product, DNA clean-up was performed using the Qiagen DNeasy PowerClean Pro Cleanup Kit. DNA was stored at -20°C until use.

Gene Amplification

Table 2: PCR Master Mix (for 16s rRNA gene amplification) components and amounts used

	Volume (ul)
10× Accuprime™ PCR Buffer II	2.5 ul
BSA (1.0mg/ml)	2.5 ul
MgCl2 (2mM)	1.0 ul
illumv4_515F (GTGCCAGCMGCCGCGGTAA) (10 μM)	0.5 ul
illumv4_806R (GGACTACHVGGGTWTCTAAT) (10 μM)	0.5 ul
molecular grade water	16.9 ul
Template DNA	1.0 ul

The PCR Master Mix listed was used for fecal samples. However, the PCR Master Mix was adjusted for the oral swab samples due to extremely low initial yields. The template DNA amount was increased from 1ul to 8ul.

The PCR conditions:

Table 3: PCR conditions for fecal samples

	Temperature	Time
Step 1	94 °C	2 min
Step 2	94°C	30 sec
Step 3	50°C	40 sec
Step 4	68°C	40 sec
Step 5	Repeat steps 2-4	24 times
Step 6	68°C	5 min
Step 7	4°C	infinite

The PCR conditions listed were used for fecal samples. Due to the low yields from oral samples, the number of cycles was adjusted from 25 to 35 cycles. Two of the oral swab samples (R21, R34) still showed inadequate PCR product on gel electrophoresis. While not ideal procedure, the samples were placed in the thermocycler for an additional 25 cycles to obtain comparable yields to the other oral samples. PCR products were inspected using gel electrophoresis. Gels were made using 1.0 g of agar powder for every 100 ml of TE buffer with 4 μl of ethidium bromide.

PCR Product Pooling & Sequencing

The remaining PCR products were purified using the AMPure XP system, and subsequently quantified using the Qubit dsDNA High Sensitivity assay kit. The Qubit measurements were used to calculate the amount of purified PCR product needed from each sample for pooling and sequencing through the MiSeq system. The manufacturer's (Illumina) standardized sequencing protocol was used.

Data Analysis

The MiSeq sequencing data was processed with the Mothur software [44]. Detailed explanation of the Mothur standard operating procedure (SOP) can be found on its dedicated website, https://mothur.org/wiki/miseq_sop/ [46]. Through this pipeline, pair-end sequences were combined. The initial phase has several steps to improve the quality of the sequencing data. Low-quality reads, sequences with poor alignment, gaps, and duplicates were removed. Additionally, chimeras were identified and removed using the chimera.uchime command. The Ribosomal Database Project (RDP) was used for taxonomic classification. For data visualization comparing microbial communities among different samples, principle coordinate analysis (PCoA) was used.

RESULTS

As expected, the PCoA data showed clustering by anatomical origin of sampling (Figure 2). Oral samples formed a clear grouping, as well as fecal samples. However, two fecal samples separated into their own clusters: S23f and S31f. Both fecal samples came from mice placed under the same conditions, housed in the home cage only (sleep control) and received an LPS injection four hours prior to removal. LPS is known to induce inflammation and increase gut permeability [47]. However, oral samples from the same pair of mice did not deviate from the rest of the mice oral samples.

Taxonomic classification of the fecal samples indicated composition that mainly included Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria in varying amounts. Taxonomic classification of oral samples showed high percentages of Firmicutes and Proteobacteria, and Actinobacteria and Bacteroidetes at minor percentages in the oral microbial communities.

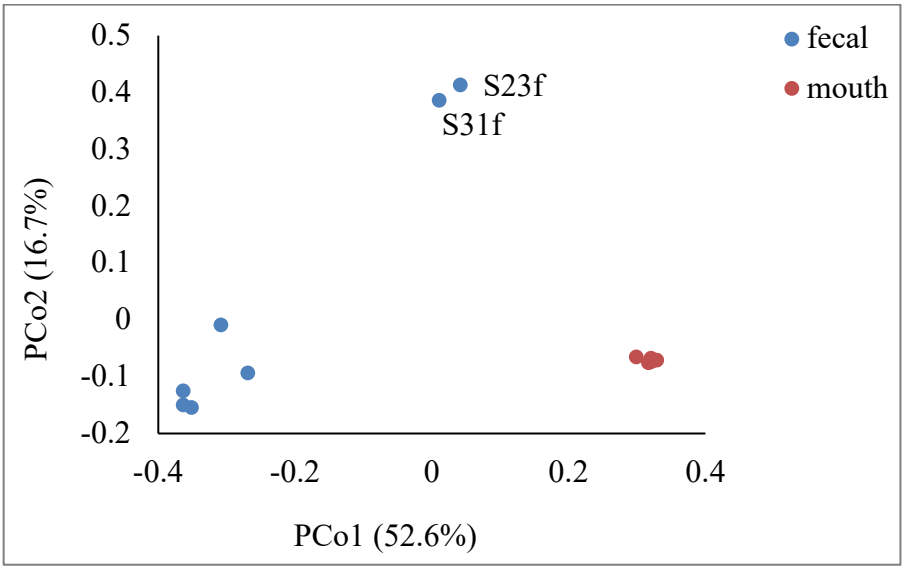

Figure 2: Principle coordinate analysis (PCoA) of the fecal and mouth microbiome profiles

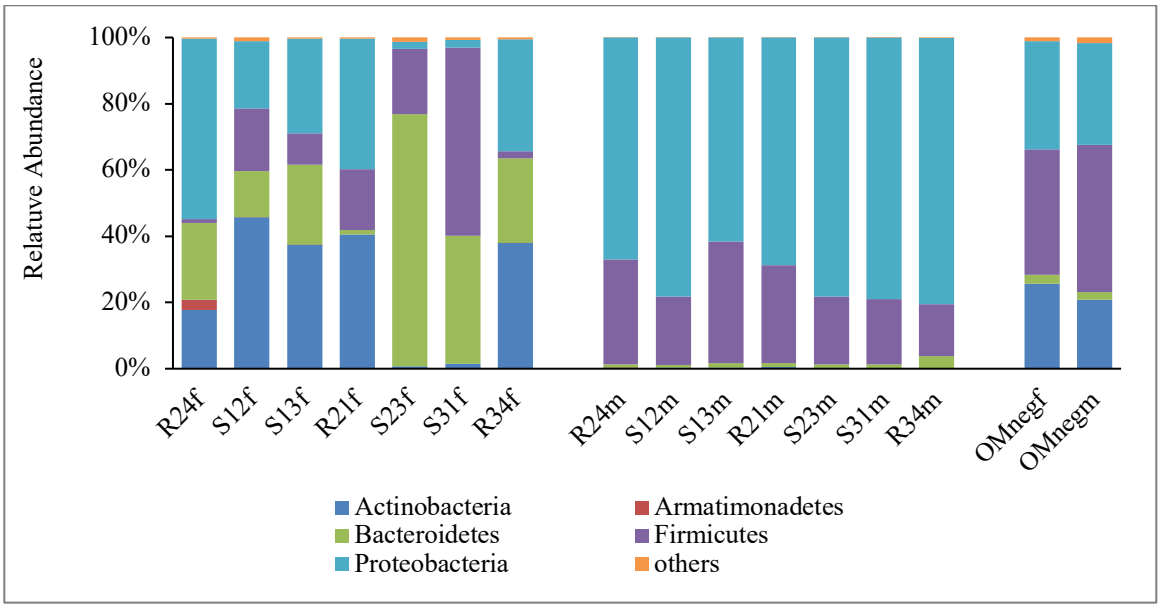

Figure 3: Murine microbiome profiles indicating the representative phylum at two different anatomical sites, gut (fecal) and mouth.

Table 4: Percentages of the taxonomic phylum compositions

Sample ID	Actinobacteria	Armatimonadetes	Bacteroidetes	Firmicutes	Proteobacteria	others
R24f	17.8%	3.0%	23.3%	1.0%	54.6%	0.3%
S12f	45.8%	0.0%	13.9%	18.9%	20.2%	1.2%
S13f	37.4%	0.0%	24.2%	9.6%	28.4%	0.4%
R21f	40.5%	0.0%	1.3%	18.4%	39.5%	0.4%
S23f	0.8%	0.0%	76.1%	19.7%	2.0%	1.3%
S31f	1.5%	0.0%	38.7%	56.8%	2.4%	0.7%
R34f	38.0%	0.0%	25.6%	2.1%	33.9%	0.4%
R24m	0.4%	0.0%	0.9%	31.7%	67.0%	0.0%
S12m	0.4%	0.0%	0.7%	20.8%	78.2%	0.0%
S13m	0.4%	0.0%	1.3%	36.7%	61.6%	0.0%
R21m	0.5%	0.0%	1.3%	29.5%	68.8%	0.0%
S23m	0.2%	0.0%	1.1%	20.4%	78.2%	0.0%
S31m	0.1%	0.0%	1.2%	19.7%	79.0%	0.0%
R34m	0.1%	0.0%	3.7%	15.6%	80.4%	0.1%
OMnegf	25.6%	0.0%	2.8%	37.9%	32.7%	1.0%
OMnegm	20.9%	0.0%	2.2%	44.5%	30.8%	1.6%

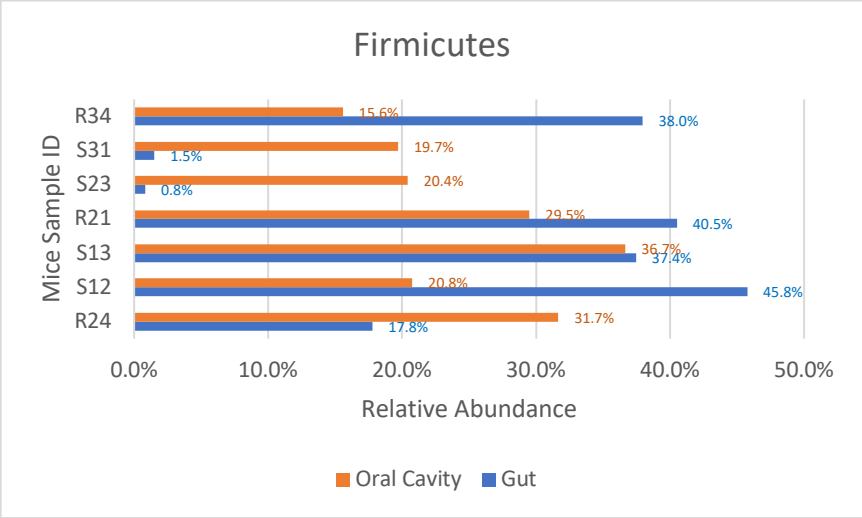

Figure 4: Firmicutes relative abundance in each sample. Noticeable deviation in both fecal samples (gut site) and oral samples of the Group C category (c/L), control sleep condition and LPS injection group (samples S23, S31).

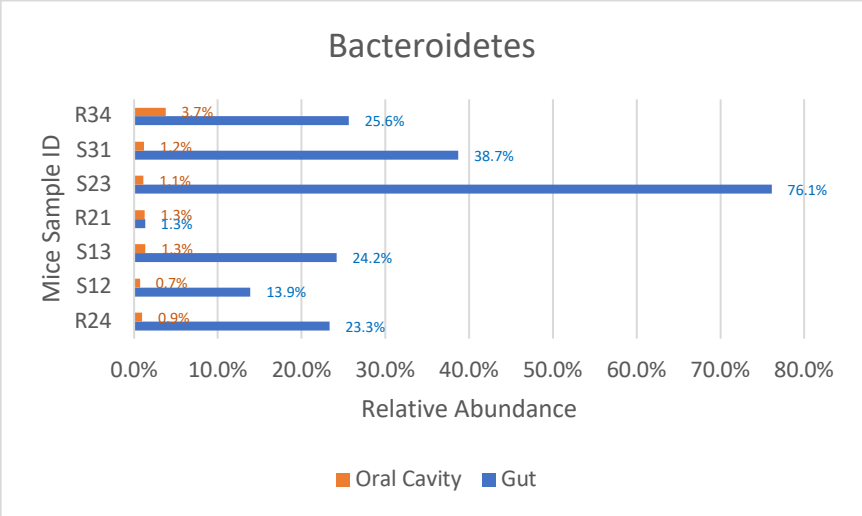

Figure 5: Bacteroidetes relative abundance in each sample.

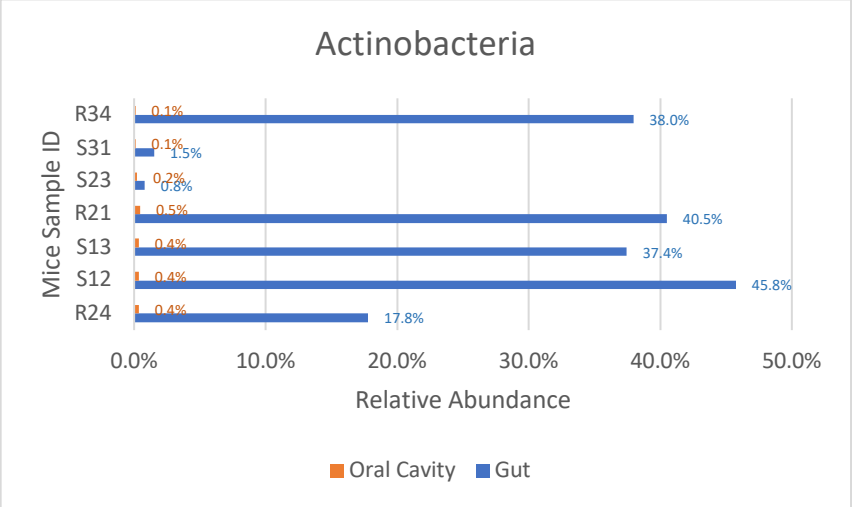

Figure 6: Actinobacteria relative abundance in each sample. Noticeable deviation in the fecal samples (gut site) of the Group C category (c/L), control sleep condition and LPS injection group (samples S23, S31).

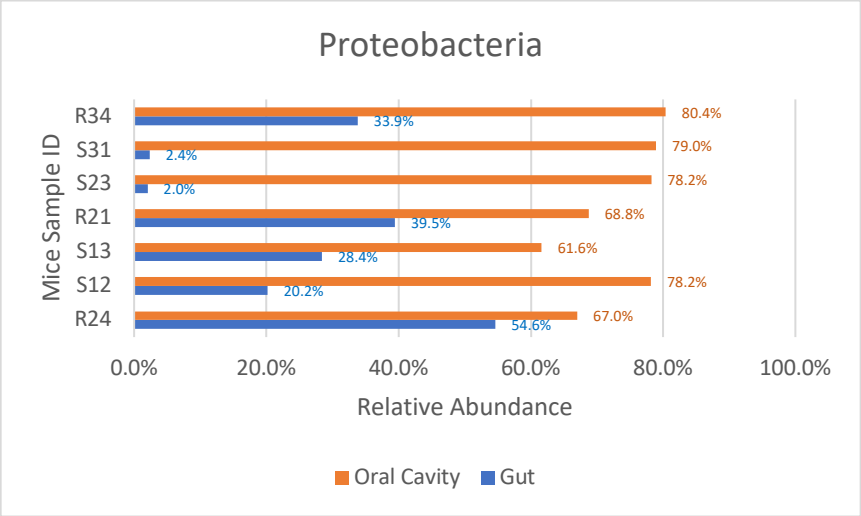

Figure 7: Proteobacteria relative abundance in each sample.

The composition of Firmicutes in the healthy mice gut is more variable than in humans, ranging from 20-80% [48]. All the fecal samples fall within the expected range of composition. Therefore, it cannot be concluded from this study that the intestinal proteobacteria has any association with stress induced by sleep deprivation. Firmicutes are also typically abundant in the oral cavity, roughly 20% [32]. Oral samples from R24 (s/s), S13, R21 (s/L) indicated increased abundance of Firmicutes compared to an expected 20%, approximately. Thus, three out of four of the sleep restricted mice ($x\bar{}$=29.7%, n=4) resulted in higher than control ($x\bar{}$=18.6%, n=3) percentages of oral Firmicutes (Wilcoxan Signed Ranked test, W=7, p=0.625). Additional studies with larger sample sets are necessary to further investigate the possible association between sleep-restricted induced stress models and increase in oral Firmicute abundance. No conclusive statement can be made on the relationship due to low sample number causing high probability the result observed is due to chance. The median difference between the pairs in the entire dataset is not indicated to be significantly different than zero (Wilcoxan Signed Ranked test, W=16, p=0.81).

Bacteroidetes is the second most abundant bacterial phylum in a healthy gut, typically ranging from 20-40% of the intestinal microbial community [48]. Fecal sample R21 (s/L) has a

markedly diminished Bacteroidetes population. However, fecal sample S23 (c/L) has pronounced increase of Bacteroidetes, nearly twice the percentage of mice model R31 (c/L) with identical experimental conditions. The sleep restricted mice ($\bar{x}$=15.7%, n=4) resulted in much lower abundance than control ($\bar{x}$=46.8%, n=3) percentages of fecal Bacteroidetes. Additionally, the differences in proportion of Bacteroidetes in the intestinal site is markedly greater than its oral cavity counterpart (Wilcoxan Signed Ranked test, W = 21, p=0.036) for all samples, except from R21 (s/L) which showed equal abundance in both oral and fecal samples.

Actinobacteria is expected to be in relatively low proportions (less than 10%) in the healthy mouse gut [48]. Group C fecal samples (S23, S31), with control sleep condition and LPS injection, had very low Actinobacteria in the gut; while the rest of the samples had greatly increased Actinobacteria in the gut. The typical oral microbiome has fairly low levels of Actinobacteria, which is reflected in the oral sample results with little variation among the different samples. The median difference between pairs is significantly different than zero (Wilcoxan Signed Ranked test, W=28, p=0.016).

Proteobacteria are present in low (less than 10%, median 2%) amounts in the healthy mice gut [48]. Group C fecal samples (S23, S31), with the control sleep condition and LPS injection, had expected proteobacteria percentages. However, the rest of the samples have greatly increased Proteobacteria abundance, including the control sample. Additional studies are necessary to investigate the possible roles stress conditions or LPS have on Proteobacteria in the intestinal microbiome. Proteobacteria percentages were consistently high for all mouth samples (>60%). Its levels have been observed to considerably increase in the post-mortem oral cavity [49] and could be a possible explanation for the high proportions of Proteobacteria detected. The median

difference between pairs is significantly different than zero (Wilcoxan Signed Ranked test, W=0, p=0.016).

DISCUSSION

General niche characteristics were exhibited across the samples in the relative abundance profiles of the oral and fecal microbiome as expected. The oral samples had less diverse range of phyla, favoring Proteobacteria and Firmicutes. The gut samples had more diverse range of phyla, and there was not favoring of any specific phyla across all fecal samples. Among the oral microbiome phylum analysis, only the Firmicutes indicated a possible association with sleep restriction. Sleep restricted mice had marked decrease in Firmicutes abundance compared to the control mice's oral samples. No associations were prevalent in the oral profiles for Bacteroidetes, Actinobacteria, or Proteobacteria in relation to sleep restriction.

Among the fecal microbiome phylum analysis, two samples consistently clustered away from the rest. These two samples (S13, S21) were categorized as group C with conditions of control home cage and LPS injection four hours prior to euthanasia. Group C samples had marked decrease in Firmicute, Actinobacteria, and Proteobacteria compared to the rest of the fecal samples. However, Bacteroidetes were greatly increased in Group C mice compared to the rest of the samples. LPS is a component of Gram-negative bacteria which provides itself a protective permeability-barrier and bacterial pathogenicity [45]. Unexpectedly, not all of the mice which received LPS injections clustered together on the PCoA data. It is possible that sleep restriction created a stress reaction in the gut towards dysbiosis. Already in a state of disequilibrium, injecting LPS in these sleep restricted models did not cause the drastic changes in phylum profile exhibited in mice receiving LPS without sleep restriction (control home cages). Another explanation may be that the six-week period of chronic sleep deprivation desensitized the host's physiological

responses to additional stress factors, like the inflammatory inducing LPS. This diminished host physiological response may align with the minimal deviations and similar gut phylum profiles among both the sleep restricted mice given LPS and saline (control). Additionally, the changed host physiological responses would affect the nature of gut microbial communities. Additional studies are necessary to further understand the complex mechanism that are at taking place.

While some associations were noted between certain phyla and the stress condition, additional studies are necessary to further investigate and confirm any correlation. This study was conducted as an extension of another major research project, with the intentions of not interfering or changing any conditions that would affect the main project. Thus, there were experimental design limitations and flaws in this study.

The major project included two variable conditions: sleep restriction vs no sleep restriction (control cage) and LPS injection vs saline injection (control), resulting in four combinations of test groups ((a) sleep restriction with LPS injection, (b) sleep restriction with control injection, (c) control cage with LPS injection, (d) control cage with control injection). This extension study looked at data from two sites: gut (fecal samples) and mouth (oral swab samples). A critical limitation of this study was the low number of samples which will be discussed later in this section. Only seven samples (n=7) were viable for study from the oral site and the seven fecal samples from the same mice specimen.

Oral swab samples and fecal collection occurred after euthanization of mice specimen to prevent introduction of any unwanted variables from this study into the major study. As such, this study only analyzed endpoint samples. Further investigations exploring the relationship of the oral and gut microbiome in stress models could benefit from collecting periodic samples over a longer duration of time. Samples collected prior to stress protocol are important to learn of the baseline

microbiome profiles. The periodic samples to follow will allow for comparison and analysis of any trends that arise from introduction of stress variable.

Additionally, mouth samples had very low biomass yields. Thus, compensatory processing steps were incorporated to improve the oral sample yields such as DNA clean-up and increasing the number of PCR cycles. Even with the aid of further processing, only seven (n=7) oral samples out of 16 had yields that were sufficient to be analyzed. The oral cavity was swabbed post euthanasia, decapitation, and cerebrum removal. Under these conditions, saliva production was halted and drying of the oral cavity was additionally increased by additional openings caused by cerebrum removal. A consideration in altering the original oral sampling technique under these conditions is to wet the swab tip with a drop of distilled water prior to swab collection. Wet swab samples have been shown to have much higher yields than dry samples [50, 51].

A significant problem with my swab samples was extremely low DNA yield causing several samples to be excluded from analysis. Since the oral samples were collected after the cerebral tissue was collected, the oral cavity likely began to dry during that time without active salivary production post-mortem. Following standard forensics collection methods [52], wetting the swab with a small amount of distilled water prior to collecting the sample might have improved the DNA yield. Additionally, gingival tissue samples were also collected during my study. However, I was unable to identify the proper methods to extract workable and specific genetic material from these samples.

The widespread issue of reduced sleep quality and sleep deprivation is a common stress factor. Such stress can lead to the development of health complications, diseases, and chronic inducement of inflammation. These stressors can have an impact on the state of the oral microbiome. For example, oral microbiota changes have been well documented relating to

periodontal disease. Continued research to better understand the role of the oral microbiome in the context of human health can ideally lead to the development of new oral screening tools. The oral cavity is a non-invasive, easy point of access for collecting samples. It is already utilized for several types of important medical testing, such as paternity, drug use, HIV, and more. Developing more tools in this area will increase access of care and help with early detection of targeted health conditions.

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