ABSTRACT

Distal Gut Microbiome Association with Sleep Duration and Quality

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The relationship between sleep time and gut microbiota composition has revealed conflicting results. Previous research has focused on the acute effect of sleep deprivation on microbial composition, however, no research has analyzed the association between habitual sleep time and gut microbial composition.

By using a cross-sectional design we evaluated whether sleep duration and quality were associated with distal gut microbiota composition in a young, healthy population under normal living conditions. A total of 55 male (n=28) and female (n=27) participants, normal BMI within 18 to 35y were recruited. Sleep and physical activity were analyzed by having the participants wearing a SenseWear® monitor for 7 days. Sleep quality was evaluated using the PSQI questionnaire. Body composition was examined through DXA. Alpha diversity was evaluated via 16S rRNA-V4 region. Metabolic syndrome risk was calculated according to the ATPIII criteria. Diet was analyzed through 24h food-records, and anxiety and depression were measured through Beck inventories.

The main hypothesis of the present study was rejected, as we did not observe a significant relationship between habitual sleep duration and alpha diversity parameters in our young, healthy, normal BMI population; results that are consistent to previous studies with lower numbers of participants showing no effect of acute sleep deprivation on alpha diversity. It is possible that the high physical activity levels of our population and/or the lack of a secondary stressor prevented us from observing an effect of sleep on alpha diversity.

Other key results observed either in female or male participants are: 1) the positive association between diet variety and gut microbial diversity, 2) the negative association between sleep quality and protein intake, 3) the stronger effect that diet has on alpha diversity in comparison to body composition, sleep, or physical activity, 4) the lower alpha diversity with higher BMI, 5) the lower alpha diversity with higher anxiety levels, 6) the relationship between poor sleep quality and depression/anxiety levels, 7) the potential mediator effect of physical activity and lean mass on the positive relationship between sleep time and fat mass, and lastly 8) the association between higher blood pressure and lower sleep time, as well as the potential mediator effect of gut diversity controlling this relationship.

Overall, this work does not support a potential relationship between sleep parameters and gut alpha diversity in young, healthy participants performing high physical activity levels.

Distal Gut Microbiome Association with Sleep Duration and Quality

by

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A Dissertation

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ABBREVIATIONS

BMI	Body Mass Index
CFS	Chronic Fatigue Syndrome
DBP	Diastolic Blood Pressure
HEI	Healthy Eating Index
LPS	Lipopolysaccharides
MVPA	Moderate to Vigorous Physical Activity
NSF	National Sleep Foundation
SBP	Sistolic Blood Pressure
TMAO	Trimethylamine N-Oxide

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DEDICATION

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CHAPTER ONE

Background

Increasing levels of obesity and the lack of successful long-term weight loss interventions have motivated scientists to identify novel ways to target obesity prevention and treatment. Gut microbiota composition and sleep are two areas that have shown to influence body composition.

The effect that the human gut microbiota has on body composition was initially discovered by performing fecal transplantation in animal models (Turnbaugh et al. 2006). Following transplantation from obese donors (ob/ob mice), a significant increase in fat mass was observed in germ-free mice, surprisingly without affecting the energy consumption of the host. This research suggests that microbiota from obese donors, which has an increased *Firmicutes* to *Bacteroidetes* ratio, may have a higher potential to harvest energy from food (Turnbaugh, Ley et al. 2006; Sze et al. 2016). The *Firmicutes* to *Bacteroidetes* ratio may be related with parameters of body composition; although this premise is still under debate (Ley et al. 2006). One of the potential mechanisms through which the microbiota could affect body composition is by altering the release of hormones related with the hunger/satiety center in the hypothalamus (Graham et al. 2015). Lifestyle factors such as diet, exercise, antibiotics use, and supplementation with pre- or probiotics have consistently been shown to modify the composition of the gut microbiota. According to recent research (Benedict et al. 2016), sleep duration might also play a key role in defining the composition of the gut microbiota and thus, alter body composition through regulation of the bacteria present in the gut.

Humans dedicate around one-third of their lifetime to sleep, highlighting the importance of having an in-depth understanding of this area of research. A healthy sleeping pattern involves both adequate duration and good sleep quality. Sleep quality refers to the absence of disrupted sleep, which can cause alterations in the sleep architecture, with little or no reduction in sleep duration (St-Onge 2013). Prior research has shown that shortened sleep duration and poor sleep quality, as well as, working on night shifts, are associated with higher cardiometabolic and diabetes risk, and higher BMI and body fat mass, especially located in the abdomen (Chaput et al. 2011b; McNeil et al. 2013; Schiavo-Cardozo et al. 2013; Iglayreger et al. 2014; Wirth et al. 2015; Mendelson et al. 2016; Poggiogalle et al. 2016). Some of the identified mechanisms through which sleep can potentially affect body composition are: 1) through increments in energy intake (especially coming from carbohydrates) caused by blunted post-meal suppression of the orexigenic hormone ghrelin and by blunting the post-meal rise of the anorexigenic hormone xenin (Schiavo-Cardozo, Lima et al. 2013; Poggiogalle, Lubrano et al. 2016); 2) by decreasing energy expenditure, and 3) by over-activation of the Hypothalamo-Pituitary-Adrenal axis (HPA) (Omisade et al. 2010; Schiavo-Cardozo, Lima et al. 2013). Another mechanism that has been recently proposed as a potential way through which sleeping alterations could affect body composition is by modifying the gut microbiota (Benedict, Vogel et al. 2016). Interestingly, the opposite direction of this relationship showing an effect of the gut microbiome on the circadian clock has also been demonstrated (Leone et al. 2015).

A recent study analyzing the effect of sleep duration on the human gut microbiota found significant alterations in response to acute sleep deprivation (Benedict, Vogel et al.

2016). This study compared two conditions of two days each; sleeping for only 4.25h/day versus sleeping 8.5h/day. The analysis revealed that after the two days of sleep deprivation the gut microbiota significantly increased in the *Firmicutes:Bacteroidetes* ratio, with a higher abundance of *Coriobacteriaceae* and *Erysipelotrichaceae*, and lower concentration of *Tenericutes*, all of which have been previously associated with metabolic perturbations in animal and human models. A similar study but with a longer sleep deprivation period failed to observe significant alterations in the gut microbiota of humans; although, it was able to show significant alterations in the gut microbiota of rats (Zhang et al. 2017).

Recent research analyzing the acute effect of sleep duration on the composition of the gut microbiota has been equivocal (Benedict, Vogel et al. 2016; Zhang, Bai et al. 2017); however, no studies to date have analyzed the long-term relationship between sleep duration and quality with gut microbiota composition. Consequently, the primary goal of the present study is to determine whether sleep variables are associated with distal gut alpha diversity in a healthy, young population under normal living conditions.

Purpose of the Study

The main purpose of this study was to test the association between gut alpha diversity and sleep duration/quality.

Hypothesis

H1: There will be a significant positive relationship between sleep time and quality and alpha diversity.

Sub-hypotheses

H1: There will be a significant relationship between dietary parameters and alpha diversity.

H2: There will be a significant relationship between body composition and metabolic syndrome parameters with alpha diversity.

H3: There will be a significant positive relationship between physical activity and alpha diversity.

H4: There will be significant relationship between anxiety and depression with alpha diversity.

H5: There will be a significant relationship between dietary parameters and sleep time and quality.

H6: There will be a significant relationship between body composition and metabolic syndrome with sleep time and quality.

H7: There will be a significant positive relationship between physical activity and sleep time and quality.

H8: There will be a significant relationship between anxiety and depression with sleep time and quality.

Delimitations

- Participants were recruited from Baylor University by flyers and emails.
- Sixty healthy male and female participants, 18-35 years old, with a BMI between 18.5-27.0 kg/m^2 were recruited.
- Participants were excluded from the study if they were smokers, following a weight loss intervention, didn't consume a mixed diet, have consumed antibiotics in the last

month, have suffered from any diarrheal or gastrointestinal infection in the last month, or were currently taking: laxatives, fiber supplements, metformin, weight loss supplements (Yohimbine, Synephrine, Ephedrine, etc.), sleep medication (such as, Ambien, Lunesta, or diphenhydramine), sleep supplements (melatonin, valerian root, l-theanine), or stimulants (caffeine, the equivalent to >4 cups of coffee, ephedrine, Adderall, Ritalin). Participants were excluded if they were pregnant or had been diagnosed with a metabolic disease (diabetes, CVD, etc.) or a psychological condition.

- All visits took place at the Exercise Nutritional Biochemical Laboratory (EBNL) in accordance with Helsinki Code after having signed university-approved informed consent documents.

Limitations

- The study utilized a convenience sample of individuals who were within Baylor University which reduces external validity to the greater population.
- Nutritional intake can influence microbiota composition, however, in order to control for this variable participants filled out a 24h food log prior to the fecal sample collection.
- Although anxiety and depression were measured, there might be other psychological variables that were not considered and that could affect the relationship between sleep and gut parameters.
- The study only recruited participants with a normal to slightly overweight BMI, translation of these results to populations with higher or lower BMI might not be possible.

- Microbiota composition can be modified by many factors. This study tried to minimize the potential effect of those identified factors through careful screening (inclusion/exclusion criteria), however, it is probable that non-yet identified factors could have modified the microbiota composition of our participants.

Assumptions

- All participants were truthful answering inclusion/exclusion criteria questions.
- Participants were expected to carefully and honestly fill out the PSQI and the Beck anxiety and depression surveys.
- All participants arrived to their second visit in a fasted state (12 hours)
- All laboratory equipment functioned properly with validity and reliability measurements being established. To minimize potential error, proper calibration and the use of trained research staff was used.
- Participants followed instructions of keeping their regular sleeping, physical activity and dietary habits through the days they wore the SenseWear^{TM} monitor.

Definitions

- DEXA – Gold standard method for body composition screening. It uses two X-ray beams of different energy levels. Rapid (6-7 min) and relatively inexpensive test that requires a low dose radiation (10% of a chest radiograph).
- SenseWear^{TM} monitor – Sleep duration and quality were objectively measured by using a SenseWear^{TM} monitor (BodyMedia Inc., Pittsburgh, PA, USA). SenseWear^{TM} armband contains built in sensors to collect data in 3 axis: heat flux, skin temperature, and galvanic skin response in 1-minute epochs.

- 24h Food – Nutritional tool used to evaluate the food consumption of a full day.
- Stool sample kits – Kits to collect and safely store stool samples at room temperature from one month up to 60 days.
- Pittsburgh Sleep Quality Index Survey – Survey that evaluates the self-reported sleep quality and disturbances over the last month. It includes 19 items that evaluate subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, daytime dysfunction, sleep disturbance, and use of sleep medications.

CHAPTER TWO

Literature Review

Introduction to Gut Microbiota

The gut microbiota is a complex community of microorganisms that live in the digestive system. Among its main functions are to degrade complex polysaccharides (i.e. cellulose, xylans, starch and inulin) and to synthetize short chain fatty acids (SCFA) (Qin et al. 2010; Salonen et al. 2014). Besides being a source of energy for colonocytes, SCFA such as butyrate, propionate and acetate, control the organization of tight junction proteins and stimulate mucin synthesis (Burger-van Paassen et al. 2009; Peng et al. 2009; Qin, Li et al. 2010). Tight junction proteins seal the intestinal lumen creating an epithelial barrier that protects the gut, while mucin maintains the integrity of the gut by forming a mucus layer that covers the intestinal epithelium against toxic agents (Burger-van Paassen, Vincent et al. 2009; Peng, Li et al. 2009). The gut microbiota is predominantly dominated by *Firmicutes* and *Bacteroidetes*, followed by *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (Jalanka-Tuovinen et al. 2011).

Dietary Variables and their Association to Gut Microbiota

The caloric content and macronutrient distribution of our diets account for the majority of alterations to the gut microbial composition (Graham, Mullen et al. 2015). Very low-calorie diets for example, have shown to significantly decrease the concentration of *A. muciniphila,* which produces oligosaccharides and SCFAs by degrading mucus (Derrien et al. 2004; Salonen and de Vos 2014).

Likewise, animal-based diets have shown to have a greater impact on the gut microbiota than plant-based diets; while animal-based diets have shown to affect 22 clusters of bacteria, plant-based diets only change 3 of them (David et al. 2014). Changes that are visible as fast as 5 days after having started each specific diet (plant-based versus animal-based), highlighting the quick adaptation of the gut microbiome to external conditions.

Besides the protein content, carbohydrate and fat consumption have also shown to affect gut microbial composition. In this regards, significant correlations have been found between carbohydrate intake and *E.rectale*, as well as, between fiber consumption and *Prevotella* (David, Maurice et al. 2014; Salonen and de Vos 2014). Similarly, the amount and the type of fat (saturated versus unsaturated) have shown to play a role in defining gut microbial composition. *Bacteroides spp*, abundance for example, has been associated specifically with polyunsaturated fatty acids intake (Lappi et al. 2013).

Not only does the food we eat feed our microbiota but it also directly provide us with new sources of bacteria. Bacteria such as *Lactococcus lactis, Pediococcus acidilactici*, and *Strephylococcus taxa* contained in food items such as cheese and cured meats, have shown to survive transit through the digestive system and become metabolically active in the gut (Martin et al. 2012; David, Maurice et al. 2014).

Besides substrate utilization, and pre-existing bacterial content in food, other factors that have shown to modulate microbial composition in the gut are: 1) pH through the GI tract, and 2) bile secretion (Salonen and de Vos 2014). The pH along the GI tract ranges from 6.0 in the right colon to 6.8 in the rectum (Bown et al. 1974). Polysaccharides from the diet are one of the strongest regulators of intestinal pH as they result in the formation

of SCFA which are acidic (Zimmer et al. 2012). Bile acids on the other hand, directly affect bacterial composition through its antibacterial effect (Kurdi et al. 2006). The higher the fat content in the diet the higher the bile excretion, which will ultimately impact microbial composition in the gut (Reddy 1981). High fat diets have shown to induce diffusion of lipopolysaccharides (LPS) and other bacterial components from the gut into the circulatory system, which creates an inflammatory state characteristic of obesity and diabetes (Moreira et al. 2012). The effect that high fat diets have on gut microbes could be acting through both the fat content from the diet as well as the fat-induced bile secretion (Salonen and de Vos 2014).

Anthropometric and Body Composition Variables and their Association to Gut Microbiota

Microbiota structure has shown to be able to affect body composition (Turnbaugh, Ley et al. 2006). One of the potential mechanisms through which the microbiota affects body composition is by altering the release of hormones related with the hunger/satiety center in the hypothalamus. Several strains of bacteria have been correlated with either orexigenic (hunger producing) or anorexigenic (satiety producing) hormones. The anorexigenic hormone leptin has been shown to be positively correlated with *Bifidobacterium* and *Lactobacillus*, with a significant negative correlation with *Clostridium*, *Bacteroides* and *Prevotella*. While, the orexigenic hormone ghrelin on the other hand, has shown to be positively correlated with *Bacteroides* and *Prevotella*, while being negatively correlated with *Bifidobacterium*, *Lactobacillus* and B. *Coccoides-Eubacterium rectale* (Queipo-Ortuno et al. 2013).

Besides the observed correlation between several strains of bacterial and appetite-regulating hormones, another proposed mechanism of action through which the microbiota can increase body fat mass is through the interaction between LPS (endotoxins found in the outer membrane of gram-negative bacteria) and the endocannabinoid system. In obesity, the gut microbiome increases gut permeability, thus, rising plasma LPS which in turn over-activate the endocannabinoid system in both the intestine and adipose tissue (Muccioli et al. 2010). These changes are reverted by prebiotic consumption, reducing adipose tissue, intestinal activity of the endocannbinoid system, improving gut barrier, and neutralizing adipogenesis (Muccioli, Naslain et al. 2010).

Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amounts (World Health Organization 2006). *Lactobacillus gasseri* is located within the genus of *Lactobacillus acidophilus* and is a well-known probiotic and a critical component of the gut flora. Supplementation with *Lactobacillus gasseri* (isolated from human breast milk) for 12 weeks has shown to decrease weight gain in rats following a high fat diet without altering their food consumption (Gibson et al. 1995). A similar study evaluating the effect of 12 weeks of supplementation with *Bifidobacterium animalis ssp Lactis* 420 (10^9 CFU) in mice receiving a high fat diet (60% energy from fat) showed a significant reduction in fat mass with a significant improvement in glucose tolerance (Stenman et al. 2014).

A multicenter, double blind, randomized, placebo-controlled intervention trial in humans evaluated the effect of consuming 200g per day of fermented milk enriched with *Lactobacillus gasseri* SBT2055. Participants were asked to maintain their habitual diet

and physical activity levels throughout the duration of the study. At the end of the 12 weeks, the experimental group showed a significant decrease in abdominal obesity and body weight in comparison to the control group which received a non-enriched fermented milk (Kadooka et al. 2010). Supplementation with a probiotic mix containing *Lactobacillus acidophilus* and *casei*, *Lactococcus lactis; Bifidobacterium bifidum* and *lactis* (a total of 2 x 10^{10} colony-forming units/day) for 8 weeks showed a significant reduction in waist circumference, waist-height ratio, and conicity index in a female overweight population (Gomes et al. 2017). However, not all studies have shown positive effects of probiotics on body composition. Consumption of 400g of low fat- probiotic enriched yogurt (200g/twice/day) during a weight loss program showed no significant effect on weight loss in comparison with the same amount of non-enriched low-fat yogurt. Nonetheless, the addition of probiotic to the low-fat yogurt caused a significant reduction in total cholesterol, low-density lipoprotein, insulin resistance, 2h postprandial glucose, and fasting insulin levels in comparison with the low fat yogurt itself (Madjd et al. 2016).

Surprisingly, not only a higher *Bifidobacterium* and *Lactobacillus* content but also the lack of bacteria in the gut have shown to beneficially affect body composition. Germ-free mice are resistant to high-fat diet induced obesity. Since gut microbiota helps to extract lipids from food, the lack of response to high-fat diets by germ-free mice is explained by the reduced capacity of their non-microbial guts to efficiently extract calories from food (Luna et al. 2015).

Physical Activity and its Association to Gut Microbiota

Aerobic exercise has shown to affect gut microbiota composition in animal models. Exercising 30 min per day, 5 days per week for 4 weeks has shown to affect the gut microbiota of mice (Almeida et al. 2016). This research study compared three conditions: control (Wistar), hypertensive (spontaneous hypertensive), and obese (fa/fa Zucker) rats. At baseline conditions, both control and hypertensive rats shared similar microbiota profiles that differed from those of obese rats. After the exercise-training period the three animal models showed differential response to exercise. A significant increase in *Allobaculum by* hypertensive rats, and *Pseudomonas* and *Lactobacillus* by obese rats, accompanied by a significant decrease in *Streptococcus* by Wistar rats, and *Aggregatibacter* and *Sutturela* by hypertensive rats were observed (Petriz et al. 2014).

Analysis of 1,493 fecal samples from the American Gut Project revealed that individuals who exercised more often have a significantly higher alpha diversity, as well as a significant elevation of certain members of the *Firmicutes* phylum (including *Faecalibacterium prausnitzii*, uncharacterized species of genus *Oscillospira*, *Lachnospira, Coprococcus*, and uncharacterized families of *Clostridiales*) (McFadzean 2014). Likewise, a cross-sectional comparison of the gut microbial composition of rugby players versus two control groups matched for BMI (>28) or age and gender (BMI $\leq$25), showed a significantly higher gut microbiota diversity and creatine kinase (indicator of muscle damage) concentration, as well as lower inflammatory status in the athletic population. In comparison with the high BMI group, rugby players had significantly higher *Firmicutes, Candidate Division TM6, Planctomycetes* and *Chloroflexi,* with lower *Bacteroidetes* concentration. In comparison with the age and gender matched group, rugby players had higher *Spirochaetes, Candidate Division TM6, Planctomycetes,*

Clamydiae and *Chloroflexi* concentration. Additionally, the rugby players and the low BMI control group had significantly higher proportions of the genus *Akkermansia* in comparison with the high BMI control group (Clarke et al. 2014). *Akkermansia muciniphilla* is a mucin degrading bacteria that has shown an inverse correlation with obesity in both mice and humans. Interestingly, even though the rugby players group had high BMI, their *Akkermansia* concentration was the same as the low BMI group (Karlsson et al. 2012; Everard et al. 2013). Is important to highlight, however, that athletes recruited by Clarke et al (2014) had a significantly higher caloric intake as well as macronutrient intake in comparison to both of the control groups. More importantly, protein consumption by athletes was positively correlated to microbial diversity. Thus, it is possible that the diet of the athletic group could have influenced the observed results in the gut microbiota of this group.

To help elucidate the relationship of exercise and gut microbial composition independently from diet, a prospective 8-week study evaluated the effect of exercise and protein consumption in three groups: exercise (aerobic and resistance exercise 3 times per week), protein (30g of whey protein per day), and exercise plus protein. Recruited participants were initially inactive individuals aged 18-40y who were predominantly overweight (body fat percentage >30%). After the 8 weeks of intervention, a trend for an increase in bacterial diversity was observed in the exercise and exercise plus protein groups but not in the protein group (Cronin et al. 2018). It is possible that a training protocol with higher intensity, frequency or duration was needed in order for the observed results to become significant. Further research in this area is needed to fully elucidate the relationship between exercise and alpha diversity.

Although exercise has shown to affect microbiota composition in a potentially favorable way (increments in *Lactobacillus* and *Bifidobacteria*), the realization of intense, prolonged endurance exercise has shown to generate a condition known as "leaky gut". This condition involves loosening of the tight junction proteins, thus, increasing the intestinal permeability and risk for infections (Clark et al. 2016). In fact, endurance athletes present a high prevalence of upper respiratory tract infections and gastrointestinal issues, including increased gut permeability and higher rates of bacterial translocation (Mach et al. 2016). The proposed mechanisms of action through which intense exercise can affect the gut microbiome is by reducing the gastrointestinal blood flow, oxygen, and nutrients at the same time of creating hyperthermia, intestinal permeability, and destruction of gut mucus. Increased gut permeability facilitates LPS translocation into the general circulation generating endotoxemia (Marlicz et al. 2015). In fact, a single long-distance triathlon has shown to be able to significantly increase circulating LPS concentration (Jeukendrup et al. 2000). It has been suggested that probiotics consumption may positively modify the gut microbiota's population and structure to protect individuals who follow exercise programs. Specifically, consumption of *Lactobacillus* and *Bifidobacterium* can aid at maintaining a state of general health, enhance immune function, decrease permeability, improve gut mucus layer, and reduce oxidative stress (Mach and Fuster-Botella 2016).

The majority of studies performed so far have focused on the effect of exercise on the gut microbiota; however, Hsu et al (Hsu et al. 2015) evaluated the influence of the gut microbiota on swimming time. The experimental study consisted on the comparison of three groups: germ-free, specific pathogen free, and *Bacteroides fragilis* gnotobiotic

mice. At the end of the study swimming time was significantly longer in the specific pathogen free mice and the *Bacteroides fragilis* gnotobiotic mice in comparison to germ-free mice. The higher endurance duration was accompanied by higher glutathione peroxidase and catalase concentrations, two potent antioxidant enzymes. Thus, the authors of the present study suggest that gut microbial composition is crucial for exercise performance and it could potentially be linked to antioxidant enzymes.

Overall, MVPA has shown to induce higher alpha diversity in animals and to be related to higher alpha diversity in humans. More experimental research in humans is needed to demonstrate a cause-effect relationship between exercise and alpha diversity.

Metabolic Syndrome and Cardiovascular Risk Factors and their Association to Gut Microbiota

Metabolic syndrome is defined by a cluster of metabolic disorders such as visceral fat accumulation, dyslipidemia, dysglycemia, and non-optimal blood pressure (Mazidi et al. 2016). It has been estimated that 20-25% of the world's adult population have metabolic syndrome, increasing three-times their likeliness to have a heart attack and two-times their likeliness to die from a heat attack or stroke in comparison to people without the syndrome (Federation 2006).

Research has recently shown that a dysbiotic gut microbiota contributes to the development of obesity-related metabolic disorders. Dietetic interventions involving higher consumption of fruits, vegetables, legumes, and whole grains (i.e. oat, buckwheat, white bean, soybean, yam, peanuts, guar gum, and pectin among other fiber types) within a caloric range of 1,000-1,600 kcal have shown promising results. A 9-week dietary intervention on 93 volunteers with central obesity resulted in significant decreases in

Enterobacteriaceae (LPS-producing bacteria) and *Desulfovibrionaceae* (sulfate-reducing bacteria), two endotoxin-producing pathogens. Gut permeability and plasma endotoxins, measured through plasma LPS-binding protein concentration, were also significantly reduced after the intervention. Similarly, plasma concentrations of CRP, TNF-alpha, and IL-6 significantly decreased, whereas adiponectin increased. Thus, a dietetic intervention targeted at improving gut microbiota composition resulted in reduction of endotoxin-producing bacteria with enhancement of intestinal barrier integrity, contributing to a reduction on circulating antigens and inflammation in a population with central obesity (Zhang et al. 2015).

It is crucial to highlight however, that not all obese individuals benefit the same from a dietetic intervention containing high fiber and whole grain products. Research suggests that is possible to identify individuals who will benefit the most from dietetic interventions by taking into account their initial concentration of *Clostridium felsineum* and *E. ruminantium. Clostridium felsineum* is a pectinolytic, butyrate-producer bacteria, whereas *E. ruminantium* (*Lachnospiraceae* family) produces mainly formic acid. The higher the concentration of these two bacteria the higher the chances the volunteer will favorably respond to a dietetic intervention. Is important to highlight that both predictive bacteria were present at a very low abundance, requiring high analytical depth to detect them, thus, revealing the importance of not overlooking the functional relevance of minor bacterial groups (Korpela et al. 2014).

Dietetic modulation not only helps adults but also children, and it is not limited to treat simple obesity but also genetically induced obesity. A dietetic intervention containing non-digestible carbohydrates (49g/day of fiber) and a 30% caloric reduction

for 30 days significantly induced weight loss and caused concomitant changes in gut microbial composition, as well as reductions in antigen load and inflammatory markers in both children with genetic and simple obesity. Reductions in aspartate aminotransferase, alanine aminotransferase, total cholesterol, LDL cholesterol, and TAG with improvements in glucose homeostasis were also observed in response to the dietetic intervention. The genetic obesity diagnosis used in the study was Prader-Willi syndrome as it is a genetic syndrome characterized by hyperphagia and low caloric expenditure that more closely simulate the factors causing simple obesity. The characteristic dysbiosis observed in the gut microbiota of children diagnosed with Prader-Willi was similar to the microbiota of children with simple obesity, showing higher production of toxins such as Trimethylamine N-oxide (TMAO) and indoxyl sulfate. Not only these groups shared similar microbiota at the beginning of the study but also responded similarly to the dietetic intervention showing an increase in the abundance of *Bifidobacterium spp*. Despite changes in microbial community composition no significant changes in gene richness and alpha diversity were observed after the intervention. Additionally, fecal transplantation of the pre-dietetic treatment to germ-free mice caused significantly higher inflammation and larger adipocytes in comparison to the fecal transplantation of the microbiota post-dietetic treatment from the same volunteer (Xiao et al. 2014). These changes demonstrate how modulation of the gut microbiota through a dietetic intervention containing high soluble and non-soluble fiber can confer protection against some of the comorbidities caused by obesity.

Probiotic interventions lasting 3 weeks to 2 months have shown anti-inflammatory effects in patients with irritable bowel syndrome and healthy adults (Spiller 2005;

Kekkonen et al. 2008). Consumption of a probiotic yogurt containing *L. acidophilus* La5 and *Bifidobacterium lactis* Bb12 by patients with diabetes mellitus for 6 weeks was found to significantly reduce fasting glycemia and hemoglobin A1c (Ejtahed et al. 2012). In fact, mice consuming a high fat diet showed probiotics to be able to inhibit bacterial infiltration in the mesenteric adipose tissue preventing diabetes onset (Amar et al. 2011).

In regards to lipidemia, probiotics have shown to decrease total and LDL cholesterol in plasma of participants with high, borderline high, and normal cholesterol levels (Guo et al. 2011).

Human research has shown that probiotic treatment (*Lactobacillus helveticus* and *Saccharomyces cerevisiae*) is able to reduce SBP and DBP in hypertensive patients (Hata et al. 1996; Khalesi et al. 2014). A proposed mechanism of action through which probiotics decrease blood pressure is by the production of ACE-inhibitor molecules through the proteolysis and fermentation of fermented milk proteins (Gonzalez-Gonzalez et al. 2013). Furthermore, a single case report demonstrated a significant decrease in both systolic (SBP) and diastolic blood pressure (DBP) when a treatment-resistant patient was treated with a combination of antibiotics (vancomycin, rifampin, and ciprofloxacin) (Qi et al. 2015) thus, suggesting a mediator role of the microbiota controlling hypertension.

Together, this data suggests the beneficial effect of caloric restriction, as well as, fiber and probiotic intake to ameliorate the negative effects of metabolic syndrome by acting through changes in the gut microbiome.

Anxiety and Depression and their Association to Gut Microbiota

The GI tract and the brain have a bidirectional communication between them known as the gut-brain axis (Foster et al. 2013). Some of the established pathways of

communication between these two organs are: the autonomic nervous system, the enteric nervous system, the neuroendocrine system, and the immune system. Recent research has suggested that commensal bacteria could be another communication pathway between the gut and the brain (Foster and McVey Neufeld 2013). The gut microbiome has the potential to modulate neurological responses such as sleep, appetite, mood and cognition (Kali 2016). Probiotics like *Lactobacillus acidophilus, Bifidobacterium infantis, Bifidobacterium longum, Candida*, and *Streptococcus* have shown to be able to produce neurotransmitters with psychotropic effects such as gamma-aminobutyric acid (GABA), glycine, serotonin, catecholamines, and acetylcholine. These neurotransmitters produced by intestinal bacteria may induce the release of neural signaling molecules by the epithelial cells thus, modulating the brain function and behavior of the host (Wall et al. 2014; Kali 2016). Another potential mechanism through which bacteria could communicate with the brain is by the activation of sensory neurons located in the mesenteric plexus as they have shown to be less excitable in germ-free mice in comparison to controls (Foster and McVey Neufeld 2013).

Animal research has shown that gut microbiota alterations produce anxiety and depression features as well as abnormal response to stress (De Palma et al. 2017). Comparison of the gut microbiota from patients with depression versus healthy controls showed significantly higher *Bacteroidales* concentration in the depression group despite no significant differences in microbial richness (Naseribafrouei et al. 2014). Specific rRNA-targeted analysis demonstrated lower *Bifidobacterium* concentration and a tendency to have lower *Lactobacillus* concentration in patients with depression (Aizawa et al. 2016). On the other hand, germ-free mice (an animal model with depleted gut

bacteria) show reduced anxiety- and depression-like behaviors and improved memory performance in comparison to controls. Interestingly, fecal transplantation from patients with depression to germ-free mice results on the appearance of depression-like behaviors. A taxonomic consortium has been identified as responsible for the differences between the "healthy microbiota" versus the "depression microbiota" of the recipient mice (Zheng et al. 2016).

Gut microbiota composition has shown to influence the release of neurotransmitters that regulate depression and anxiety in the central nervous system. Furthermore, human research has shown that probiotics are able to reduce anxiety, depression, and hyper-reactivity by acting through the gut microbiota (Slyepchenko et al. 2014; Wallace et al. 2017).

Introduction to Sleep

According to the National Sleep Foundation (NSF) the recommended sleep time for young (18-25y) and adult populations (26-64y) is 7-9h per day (Foundation 2015). However, despite these recommendations the prevalence of sleep-related problems have increased from 62% in 1999 to 75% in 2005 (Foundation 2005b). According to the NSF, 61% of adolescents (13-18y) sleep less than 8 hours during weekdays, with 46% rarely getting a good night sleep (Foundation 2011). The most important factors that have contributed to the decrease in the sleeping duration observed in adolescents and young adults are: the existence of electronic devices in bedrooms as well as the presence of emotional and academic stress (Lund et al. 2010; Foundation 2012). It is estimated that around 89% of adults and 75% of children have at least one electronic device in their bedrooms, which is significantly associated with lower sleep time. Additionally, it has

been shown that whenever these devices are left on during the night, they cause lower sleep quality which has shown to have similar effects on general metabolism as actual sleep loss (Baud et al. 2013; Foundation 2014). Recognizing the importance of sleep is crucial as research continues to show an increased mortality in poor sleepers, especially when combined with simultaneous stressors (Krueger et al. 2016).

Normal human sleep architecture is formed by two states, Rapid Eye Movement (REM or R) and Non-Rapid Eye Movement (NREM or N), which alternate across the sleeping period. NREM state comprises 75-80% of the sleeping time, and is characterized by slow waves and low muscle tonus as well as minimal psychological activity. NREM is defined as a relatively inactive state in a movable body. On the other hand, REM state consists of around 20-25% of the sleeping time and is characterized by atonic muscles and the presence of dreams. It can be defined as having an active brain in a paralyzed body. Both, NREM and REM states cycle every 90 minutes. According to the NSF, sleep normally begins in NREM and progresses into its different stages (N1 to N3), which last from 80-100 minutes. NREM is followed by REM, which is not divided into stages. N1 of NREM (formerly stage 1) lasts for 1-7 minutes and has a low arousal threshold, in other words, it can be easily discontinued by external stimuli. N2 of NREM (formerly stage 2) comprehends a period of 10-25 minutes and has a higher arousal threshold, which means that a more intense stimulus is required in order to produce a response. N3 (formerly stage 3 and 4) of NREM lasts only a few minutes and is known as slow wave sleep. In order to produce a response in this last stage an even larger stimulus would be needed in comparison with N1 or N2. REM sleep lasts only 1-5 minutes in the first cycle; however, its duration increases every time a REM episode takes place. There are usually

4-6 discrete REM episodes throughout the night. Arousal stimulus that take place during REM state are believed to be usually incorporated into the ongoing dream story rather than producing an awaking response (Carskadon et al. 2011).

The circadian system provides temporal organization of the body's function within a period of 24h and is controlled by the suprachiasmatic nucleus, located in the anterior hypothalamus. Several hormones are involved in sleep and circadian rhythmicity such as growth hormone, cortisol, and melatonin. Melatonin and cortisol exhibit robust circadian rhythmicity. Melatonin, a hormone produced by the pineal gland, has an overall higher concentration during night versus day, while cortisol, a hormone produced by the adrenal glands, peaks right before morning. Growth hormone, produced by the anterior pituitary, increases during sleep and peaks immediately after sleep onset. However, under circadian disruption, such as the one observed during sleep deprivation, there is a change in the secretion of these hormones resulting in an increase in cortisol with a reduction in leptin, melatonin and thyrotropin release, as well as an altered ghrelin (orexigenic hormone) and xenin (appetite suppressant hormone) secretion (Crowley et al. 2007; Schiavo-Cardozo, Lima et al. 2013; Kim et al. 2015). Some of these hormones (leptin and ghrelin) have previously shown to be correlated to specific strains of bacteria in the gut. Specifically, leptin has shown a positive association with *Lactobacillus* and *Bifidobacteria*, whereas Ghrelin has demonstrated a negative association with these two bacterial strains (Queipo-Ortuno, Seoane et al. 2013).

Dietary Variables and their Association to Sleep

Sleep deprivation and fragmentation have shown to increase hunger and energy intake predominantly from carbohydrates and fat (Nedeltcheva et al. 2010; Doo et al.

2016; Poroyko et al. 2016). A large, nationally-representative study demonstrated that both women and men that sleep less than 7h consume less protein, with a higher consumption of fat for men and carbohydrates for women (Doo and Kim 2016). Working during the night has also shown to cause a significant increase in energy intake coming mainly from carbohydrates and fat (Schiavo-Cardozo, Lima et al. 2013; Xiao et al. 2013).

Anthropometric and Body Composition Variables and their Association to Sleep

It is well known that obesity causes sleeping alterations (i.e. obstructive sleep apnea) and that sleeping alterations can in turn increase obesity risk, resulting in a vicious cycle (Chaput, Despres et al. 2011b; Drager et al. 2013; Xiao, Arem et al. 2013; Iglayreger, Peterson et al. 2014). To make the relationship even more complex, it has been shown that not only short-sleepers (5-6h) but also long-sleepers (9-10h) suffer higher weight gain in comparison with average-sleepers (7-8h) over a period of 6 years (Chaput et al. 2008; Magee et al. 2010; Xiao, Arem et al. 2013). This U-shaped relationship suggests an optimal sleeping time of 7-8h per day. In addition, short and long sleepers have a 58% and a 47% higher increase in waist circumference, respectively, and a 124% and a 94% higher body fat percentage in comparison with average sleepers (Chaput, Despres et al. 2008; Xiao, Arem et al. 2013). Research has also shown that female participants sleeping on average 8.5h had a mean body fat similar to those who slept <6h. The lowest percentage of body fat measured by BODPOD was detected in those sleeping 8.0-8.5h per night (Bailey et al. 2014).

Furthermore, short sleep duration doesn't directly increase weight gain *per se* as research suggest that the presence of a high disinhibition eating behavior is needed in order to increase weight and abdominal circumference in short sleepers (Chaput et al.

2011a). This observation was the result of a 6-year longitudinal study in which participants who sleep short and presented high disinhibition scores had a 2.5 higher incidence of being overweight/obese in comparison to those participants who presented short sleep but low disinhibition scores. A disinhibited eating behavior represents a tendency towards overeating in response to cognitive or emotional cues (Chaput, Despres et al. 2011a).

Not only can sleep deprivation and poor sleep quality increase weight gain but it can also decrease the rate of weight loss in response to a weight loss intervention (Thomson et al. 2012). Short sleep affects loss of fat mass through increased hunger, reduced energy expenditure, increased cortisol, and potentially altered gut microbiota composition. These factors can disrupt adherence to caloric restrictions and can decrease the weight loss and/or increase weight regain after a weight loss intervention. In this regards, significant differences have been identified in the composition of the weight loss between short (5.5h) versus average sleepers (8.5h), with the former ones reducing 55% less FM and 60% more FFM than the later ones (Nedeltcheva, Kilkus et al. 2010).

In addition to sleep time, having a large daily variation in one's sleep schedule can also influence fat mass. Participants with a 7-day standard deviation of >90 minutes had more body fat mass than those who had a sleeping variation of <60 min (Bailey, Allen et al. 2014). It is possible that the lack of consistency in the sleeping patterns cause dysregulation of the circadian rhythm similarly than sleep deprivation.

Sleep quality is also an important factor predicting the effect of sleep on body fat mass as it has been shown that poor sleep quality is correlated with an increased risk of having overweight/obesity (Quick et al. 2014). According to this research college-age

(18-24y) participants with a varied BMI (normal to obese) showed sleep quality measured through PSQI survey to be a significant predictor of overweight. Important to highlight that 46% of the participants (n=1252) presented low physical activity levels.

In sum, poor sleep quality, sleep deprivation and high day to day sleep variation can potentially affect body composition through: 1) higher energy intake caused by altered post-meal ghrelin and xenin release (Schiavo-Cardozo, Lima et al. 2013; Poggiogalle, Lubrano et al. 2016; Poroyko, Carreras et al. 2016); 2) decrements in energy expenditure accompanied by changes in substrate utilization (Nedeltcheva, Kilkus et al. 2010), 3) HPA over activation (Omisade, Buxton et al. 2010; Schiavo-Cardozo, Lima et al. 2013), and 4) potential alterations in the gut microbiota (Benedict, Vogel et al. 2016). While increased sleep time can affect body composition by decreasing physical activity time (Hart et al. 2017).

Sleep and Exercise

Physical activity improves subjective and objective sleep. A recent meta-analysis of 66 studies evaluated the effect of acute versus chronic exercise on sleep variables. Results revealed that acute exercise interventions have a small but beneficial effect on total sleep time, sleep onset latency, sleep efficiency, stage 1 sleep, and slow wave sleep, with a moderate beneficial effect on wake time after sleep onset. On the other hand, chronic exercise revealed to have small beneficial effects on total sleep time, sleep efficiency, and sleep onset latency with a moderate beneficial effect on sleep quality (Kredlow et al. 2015). Some of the proposed mechanisms that explain the benefits of exercise on sleep time and quality are: CNS fatigue, changes in body temperature, increased energy consumption, changes in mood/anxiety symptoms, growth hormone secretion, secretion

of brain derived neurotropic factor (BDNF), improved fitness level, and change in body composition in response to exercise. Furthermore, the moderators that regulate the effect of exercise on sleep parameters are: age, sex, baseline physical activity levels, exercise type, time of day, adherence, and duration (Kredlow, Capozzoli et al. 2015).

The relationship between physical activity and sleep seems to be bi-directional. Human research has shown that the experimental reduction in sleeping time decreases physical activity and increases time spent in sedentary activities such as watching TV (Hart, Hawley et al. 2017).

Sleep time and quality, as well as physical activity levels are strong predictors of body fat mass. Multivariate structural equation modeling has shown that a model including sleep efficiency, sleep patterns inconsistency, and physical activity is the best model to predict percent of body fat (Bailey, Allen et al. 2014).

Overall, exercise and sleep have a close bi-directional relationship in which physical activity facilitates sleep time and quality, and adequate sleep enables adequate physical activity levels.

Sleep and Metabolic Syndrome

Short sleep duration causes metabolic dysregulation increasing metabolic syndrome risk (Fernandez-Mendoza et al. 2017). In fact, not only short sleep duration but also insomnia symptoms, such as difficulty initiating sleep and difficulty maintaining sleep, have independently been correlated with metabolic syndrome (Lin et al. 2016). A 4-years longitudinal study evaluating self-reported sleep duration, found a significant higher risk of metabolic syndrome incidence in those individuals whose sleep duration decreased by $\geq$2h per night in comparison to those consistently sleeping 7h through the duration of the

study (Song et al. 2016). A similar study analyzing self-reported sleep duration in 615 adolescents found a significant correlation between adolescents sleeping <8h and centripetal distribution of fat as well as decreased insulin sensitivity (De Bernardi Rodrigues et al. 2016). In addition to short sleep duration, shift workers have higher insulin resistance, triglycerides, and CRP concentration in comparison to day workers (Schiavo-Cardozo, Lima et al. 2013).

Not only sleep duration but also sleep fragmentation has shown to affect metabolic outcomes. Animal research has shown that 4 weeks of sleep fragmentation is sufficient to induce alterations in insulin sensitivity as well as systemic and white adipose tissue inflammation that reverts after 2 weeks of normal sleep. Visceral white adipose tissue increases neutrophils, macrophages (with M1 polarity), LBP, NGAL, and IL-6 after 4 weeks of sleep fragmentation. NGAL is a glycoprotein induced by a pro-inflammatory stimulus such as the presence of LPS or other bacterial products, whereas LBP is released by various cells including macrophages located in adipocytes. This inflammatory response in visceral white adipose tissue suggests a potential transfer of gut microbial products into the circulatory system eliciting an inflammatory response (Poroyko, Carreras et al. 2016). In fact, previous animal research has shown an increased intestinal permeability caused by sleep deprivation. Effect that is reverted after chronic supplementation with *Lactobacillus plantarum* MTCC 9510 (Dhaliwal et al. 2018).

Sleep abnormalities, such as sleep apnea, have shown to significantly increase blood pressure. Sleep apnea is characterized by repeated collapse of the upper airway during sleep that leads to hypoxia and hypercapnia. Animal research has shown that although sleep apnea is not able to affect blood pressure per se, it does significantly increases

blood pressure when administered in combination with a high fat diet. Fourteen days of sleep apnea after 3 weeks of following a high fat diet showed a synergistic increase in blood pressure of up to 29 mmHg (Durgan et al. 2016). The combination of sleep apnea and high fat diet alters microbial composition in the gut by decreasing bacteria known to produce SCFA (*Eubacterium* and *Clostridiales* such as *Ruminococcaceae*) and increasing bacteria known to produce lactate (*Lactococcus* and *Coriobacteriaceae*). Microbial composition in the gut can be considered a causal factor of the relationship between sleep apnea and hypertension as fecal transplantation from hypertensive rats into normotensive rats results in hypertension after 14 days of induced sleep apnea (no effects were observed with fecal transplantation alone). Additionally, co-administration of antibiotics prevented the increase in blood pressure in rats under a high fat diet undergoing sleep apnea (Durgan, Ganesh et al. 2016). These results demonstrate the role of the microbiota on the effect of sleep apnea on hypertension. The mechanism of action between the connection of sleep apnea and hypertension seems to be the following: apnea induces intermittent hypoxia/re-oxygenation cycles in the blood and lumen of small intestine, which has shown to increase oxidative stress, up-regulation of hypoxia-inducible factor 1-alpha, down-regulation of tight junction proteins (affecting gut barrier), increment in plasma LPS and systemic inflammation. Among these factors it is plausible that intermittent hypoxia could theoretically increase the concentration of obligate anaerobes (Santisteban et al. 2016; Durgan 2017). It is possible that the presence of a second stressor, besides sleep deprivation/fragmentation, is needed in order to elicit noticeable detrimental effects, such as increases in blood pressure.

In summary, sleep deprivation has shown to increase metabolic syndrome risk by increasing insulin resistance, triglycerides concentration, inflammatory levels, and blood pressure. Alteration of gut microbial composition and intestinal permeability are two proposed mechanisms of action through which sleep deprivation influences metabolic outcomes.

Sleep and Psychological Variables

Although conflicting research exists, studies have shown the predecessor effect of sleep abnormalities on depression and anxiety. College students with depressive symptoms who also experience sleep disturbance have significantly higher anxiety levels, poorer cognitive and physical functioning, and greater impairment to remember information; in comparison to students with depression who don't experience sleep disturbances (Nyer et al. 2013).

A nationally representative study involving 9,683 young women (22-25y) analyzed sleeping patterns and subsequent appearance of depression and anxiety symptoms in follow-up surveys (3,6 and 9 years later). Results demonstrate that sleep difficulties in women who did not previously experience mental health problems, strongly predicted subsequent appearance of depression, yielding a 4-fold increased risk. Likewise, self-reported sleeping problems were able to predict a two-fold increase risk for anxiety later in life (Jackson et al. 2014). It is likely, however, that the relationship between sleep and psychological variables also possess a bidirectional effect. Twin and siblings research has demonstrated the substantial overlap between genes controlling for sleep, anxiety and depression. This genetic overlap indicates that the associations between these three parameters are mainly genetically controlled (Gregory et al. 2011).

High physical activity and low screen time in college students have independently been associated with significantly lower risks for poor sleep quality, anxiety and depression (Feng et al. 2014; Wu et al. 2015). Thus, is possible that increasing physical activity levels and decreasing screen time could prevent the appearance and development of poor sleep quality, anxiety and depression.

In sum, sleep disruption seems to precede the appearance of anxiety and depression, however, a bi-directional relationship between these variables has also been proposed. Poor sleep quality has shown to accentuate the detrimental effects of depression on anxiety, as well as, cognitive and physical outcomes, as these three factors seem to have a strong genetic overlap. Physical activity could help to improve sleep quality, anxiety, and depression.

Others: Sleep and Cortisol

Cortisol shows a daily pattern governed by the suprachiasmatic nucleus of the hypothalamus (Chung, Son, & Kim, 2011; Mohawk & Takahashi, 2011). Corticotropin-releasing hormone (CRH) produced by the hypothalamus, is released in response to a stressor. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH increases in turn the secretion of glucocorticoid hormones, such as cortisol, from the adrenal cortex (Balbo et al. 2010). The daily circadian release of cortisol starts with an "acrophase" characterized by high cortisol levels in the early morning, followed by a decline throughout the day known as "quiescent period". Cortisol concentration is centered around midnight in a stage known as "nadir", followed by a rapid rise during the second half of the night (Balbo, Leproult et al. 2010).

Longitudinal research has shown that short sleep duration (<6h) increases abdominal adiposity (Chaput, Despres et al. 2011b), which could be caused by hyper-activation of the HPA axis. Sleep deprivation is sensed by the body as a stressor due to the extra effort of the body to maintain wakefulness, thus, increasing cortisol release (Balbo, Leproult et al. 2010). A single night of sleep deprivation has shown to be enough to alter the circadian cycle of cortisol, decreasing its morning concentrations and increasing both the afternoon and evening concentration (Omisade, Buxton et al. 2010). Low cortisol concentration in the morning could potentially affect morning arousal while increased concentrations at night can affect sleep onset (Balbo, Leproult et al. 2010; Leproult et al. 2010). Nonetheless, cortisol release in response to sleep deprivation has shown mixed results, with some studies showing an increased release in response to sleep deprivation, with others showing either no change or a lower release. A potential cause of this variation could be due to the biphasic response of cortisol in response to sleep deprivation. It seems that the HPA axis is activated early in response to stress in order to adapt to sleep loss and keep the body awake; however, after a prolonged period of time the increased sleep pressure might end up blunting the HPA activity, thus, decreasing cortisol release (Balbo, Leproult et al. 2010).

In this regard, weeks of chronic circadian misalignment have shown to have a differential response in comparison to acute sleep deprivation. Circadian misalignment results when the internal clock is not properly aligned to the peripheral clock, such as when wakefulness occurs when the internal circadian clock is promoting to sleep (Gronfier, Wright, Kronauer, & Czeisler, 2007). A recent study, involving 17 healthy, drug-free males and females (20-41y), compared the acute effect of sleep deprivation

versus the chronic effect of circadian misalignment. Acute sleep deprivation consisted of keeping participants awake for 40h within the laboratory setting, whereas chronic circadian misalignment was created by keeping day cycles of 24.6h. In other words, although the allowed sleep time was kept constant (~8h) the time at which the participants were allowed to sleep shifted for 36 minutes every day. At the end of study the acute sleep deprivation caused a significant increase in cortisol concentration and psychological stress, whereas the chronic circadian misalignment caused a significant decrease in cortisol levels with no change in stress ratings across the study. Chronic circadian misalignment significantly increased the pro-inflammatory proteins tumor necrosis factor-alpha (TNF-α) and C-reactive protein (CRP), as well as the anti-inflammatory cytokine Interleukin 10 (IL-10) (Wright et al. 2015). The end effect of the concurrent increment of these two different types of inflammatory/anti-inflammatory proteins is unclear. Is possible, however, that this type of induced-sleep misalignment represents more closely sleep disorders and not other sleep-affecting conditions such as shift work as it has been shown that long-term shift workers have significantly higher cortisol concentrations in comparison to day workers (Manenschijn, van Kruysbergen, de Jong, Koper, & van Rossum, 2011).

Not only does cortisol concentration depend on whether the stress is acute or chronic but cortisol effects in the body will also greatly depend on the concurrent concentration of other hormones. Cortisol has shown to possess both lipolytic and lipogenic effects depending on the physiological state of the body. In the fasting state, glucocorticoids stimulate lipolysis and switch off lipogenesis (inactivating Acetyl–CoA Carboxylase and increasing serum FFA concentration), however, in the fed state insulin and

glucocorticoids work together to promote lipid storage (activating Acetyl–CoA Carboxylase and Fatty Acid Synthase), especially in specific fat depots such as the ones located in the abdomen and the face (Gathercole et al., 2011; Wang, Gray, Kuo, & Harris, 2012).

Effect of Sleep on Gut Microbial Composition

Circadian rhythm disruption has shown to generate microbial dysbiosis which seems to mediate the detrimental effects of sleep deprivation on metabolic outcomes. Shift workers are exposed to circadian disruption, which can detrimentally impact health-related metabolic parameters. A recent animal study evaluated the effect of chronic circadian disruption and high-fat, high-sugar diets on the intestinal microbiome. Authors created four comparison groups: 1) Non-Shifted, Standard Chow Diet, 2) Shifted, Standard Chow Diet, 3) Non-Shifted, High-Fat, High-Sugar Diet and 4) Shifted, High-Fat, High-Sugar Diet. Circadian shifts were created by weekly reversals of the light:dark cycle for a period of 12 weeks. After the experimental period, results demonstrated that regardless of the circadian status, mice under high-fat, high-sugar diets have an overt effect on microbial composition, which was characterized by significant increases in the relative abundance of bacteria from the phyla *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia*, with a concomitant reduction in the average relative abundance of bacteria from the phylum *Bacteroidetes*. Circadian disruption did not significantly affect the microbial composition *per se* unless it was accompanied by a high-fat, high-sugar diet, in which case it would cause a differential microbial change than the one caused by the high-fat, high-sugar diet itself. Despite the lack of microbial composition changes caused by circadian disruption alone, a significant increase in body weight was observed

in the circadian shifted groups. This increase in body weight was present even when circadian shifted groups consumed less food in comparison to the non-shifted groups (Voigt et al. 2014). It seems that circadian disorganization impacts the intestinal microbiome in high-fat, high-sugar fed mice, but not in mice consuming a normal diet. It is possible that a secondary stressor is needed in order for circadian disorganization to influence the gut microbiota.

A similar animal study evaluated the effect of mutated circadian clock gene on the gut microbial community (measured by 16S rRNA). Mice with the circadian clock gene mutation have features that resemblance mice subjected to environmental circadian rhythm disruption (caused by changes in the light:dark cycle). Wild type and mutated mice were fed one of the following three diets forming a total of six groups: 1) Standard Chow Diet, 2) Alcohol-Containing Diet, or 3) Alcohol-Control Diet in which alcohol calories were replaced with dextrose. Mutation of the circadian clock gene resulted in significantly lower diversity and evenness (at the genus, family, order and class level) with no change in microbial richness. Despite these changes, no significant differences in microbial composition were observed between wild type and circadian gene mutated mice consuming the Standard Diet. Presence of both, Circadian Clock gene mutation and Alcohol-Containing diet caused a significant change in the microbial composition that was different to the effects caused by Alcohol-Containing diet on Wild type mice (Voigt et al. 2016). These results suggest that mutation of the circadian clock gene perturbs the normal intestinal microbiota, however, it seems that there is a need for a secondary insult, such as a dietetic stressor, in order to exacerbate the effect of circadian disruption on the microbial composition.

Not only shifts and circadian misalignments can affect the gut microbiota but also sleep fragmentation. Animal research has shown that sleep fragmentation, such as the one observed in sleep apnea, induces gut microbial changes characterized by significant increments in *Lachnospiraceae*, *Ruminococcaceae* and *Clostridiales* with a significant decrease (2-fold) in *Lactobacillaceae* and *Bifidobacteriaceae* families. A change in the *Firmicutes* to *Bacteroidetes* ratio was also observed in response to sleep fragmentation going from 1.0 in the control group to 1.7 in the experimental group (Poroyko, Carreras et al. 2016).

A recent study analyzing the effect of sleeping duration on the human gut microbiota found significant alterations in response to sleep deprivation. The study compared two conditions of two days each, sleeping for only 4.25h/day versus sleeping 8.5h/day. The analysis revealed that after sleep deprivation the gut microbiota showed a significant increase in *Firmicutes:Bacteroidetes* ratio, a significantly higher abundance of *Coriobacteriaceae* and *Erysipelotrichaceae*, and a lower concentration of *Tenericutes*, which have been previously associated with metabolic perturbations in animal or human models (Benedict, Vogel et al. 2016). A similar study, but with a longer sleep deprivation period (4h/night for 5 days), failed to observe significant alterations in the human gut microbiota. In a different branch within the same study, similar sleep deprivation conditions were applied to rats (4h/night for 7 days). The animal data demonstrated a significant alteration in a single OTU (TM7-3a), which was found to increase in response to sleep restriction. Authors stated that there is a possibility that human microbiome dysbiosis might appear in response to chronic sleep restriction on a timescale of weeks or

months (not days), which would be a more accurate conversion to human lifespan of the sleep deprivation timeline used in the animal protocol (Zhang, Bai et al. 2017).

In summary, sleep deprivation, sleep fragmentation, and circadian disruption have independently shown to directly or indirectly alter gut microbial composition. Some studies reveal the need for a secondary stressor such as a high fat, high sugar diet, in order for dysbiosis to occur as a response to altered sleep, however, research in this area is inconsistent.

Effect of Gut Microbial Composition on Sleep

The gut-brain axis represents a bidirectional communication between the central nervous system and the gut; the communication between sleep and gut microbiome seems to also follow a bidirectional approach. Research has shown the potential for sleep to modulate gut microbial structure, however, it seems that the gut microbiome is also able to affect sleep patterns (Leone, Gibbons et al. 2015). To understand the effect of gut microbial composition on sleep patterns it is important to understand bacterial anatomy and physiology in the gastrointestinal tract. Bacteria have an inner membrane known as plasma membrane, as well as an external membrane composed by peptidoglycans. Gram-negative bacteria, have an outer membrane formed by a lipid bilayer of phospholipids and lipoproteins in the inner leaflet with LPS on the outer side. Gram-positive bacteria lack outer membranes but have components such as peptidoglycans attached to the membrane. As bacterial cycle take place, the peptidoglycans and LPS of their membranes are degraded by bacterial enzymes. Host macrophages and neutrophils can digest peptidoglycans producing muramyl peptides which have been shown to be able to translocate the intestinal wall and produce somnogenic effects (Krueger and Opp 2016).

Besides the somnogenic effect of peptidoglycans and LPS once converted into muramyl peptides, a recent animal study suggests a more intertwined connection between gut microbiota and circadian rhythm. Specifically, the study demonstrated that gut microbiota exhibits diurnal variations in composition and metabolite production that are partially driven by diet (Leone, Gibbons et al. 2015). Consumption of a high-fat diet creates a significant alteration in the normal diurnal variation of gut microbial structure and function thus, causing changes in SCFA production. SCFA directly modulate circadian clock gene expression within hepatocytes. Therefore, high-fat diets affect circadian clock gene expression in the hepatocytes by acting through alterations in the normal diurnal variations of the structure and function of the gut microbiota. Furthermore, the role of gut microbiota mediating the effects of high-fat diet on circadian gene expression is confirmed by the lack of effect of high-fat diets on circadian gene expression when using germ-free mice (Leone, Gibbons et al. 2015).

Human research suggests has shown the effect of microbiota on sleep disturbances such as Chronic Fatigue Syndrome (CFS). CFS is characterized by sleep disturbances, persistent and relapsing fatigue, constant cognitive and mood changes, gastrointestinal disturbances and food intolerances. *Streptococcus* is a bacterial strain that is thought to mediate (at least in part) the effect of gut microbiota on CFS. In a recent study, twenty-two participants were provided with 400 mg of the antibiotic erythromycin for 6 days. From the 22 participants, 13 had a reduction in *Streptococcus* concentration at the end of the study but only 7 participants achieved a final concentration of <6%. While other 4 participants observed an increase in *Streptococcus* concentration and 4 others did not see a change. At the end of the study, total sleep time increased by 44min from baseline in

the responders group, while sleep time decreased 15 min in the non-responders group. Authors recognize, however, that even though antibiotic treatment decreased *Streptococcus* concentration in some patients, it also significantly decreased *Lactobacillus* and *Bifidobacteria*, which are thought to have a beneficial effect. Combination of antibiotics with probiotics might have a stronger effect on regulating the potential influence of the microbiome on sleep disorders such as CFS (Jackson et al. 2015). Although more research is needed, it seems that the gut microbiome can in turn affect sleep patterns contributing to this bidirectional communication between the gut and the CNS.

Study Rationale

Recent research has shown that only 29.4% of college students satisfy the recommended sleeping time of 8h or more, with 25% getting less than 6.5h (Lund, Reider et al. 2010). Likewise, it has been estimated that around 60% of adolescents fall within the poor-quality sleep category from the PSQI, with the most important factors affecting these alarming levels being emotional and academic stress. This population is at higher risk to develop sleep abnormalities due to the lack of adult supervision, inconsistent schedules, increased homework, extra-curricular activities, part-time jobs, and the high use of TV, video games, computers, and smartphones at late hours (Cain et al. 2010; Lund, Reider et al. 2010). Furthermore, not only external factors can affect the sleeping patterns of adolescents but also internal ones, such as a physiologic delay in the circadian phase present in adolescents (Crowley, Acebo et al. 2007). It has been demonstrated that bedtime and rise-times are shifted by around 90 minutes from weekdays to weekends in this population, behavior that seems to extend into adulthood (Lund, Reider et al. 2010).

Adequate sleep is fundamental to prevent detrimental effects on energy intake (Nedeltcheva, Kilkus et al. 2010), physical activity (Hart, Hawley et al. 2017), body composition (Chaput, Despres et al. 2008), insulin resistance (Poroyko, Carreras et al. 2016), lipid profile (Rebolledo et al. 2017), blood pressure (Durgan, Ganesh et al. 2016), anxiety, and depression (Nyer, Farabaugh et al. 2013) among other health-related variables. Gut microbiota has been proposed as the mechanism of action through which sleep influences metabolic outcomes (Voigt, Forsyth et al. 2014; Benedict, Vogel et al. 2016; Voigt, Summa et al. 2016). It seems that an altered gut microbiota causes increments in gut permeability (Zhang, Yin et al. 2015), which creates bacterial translocation, increasing circulating LPS and generating inflammation (Santisteban, Kim et al. 2016; Durgan 2017). Physical activity, caloric restriction, increased fiber and probiotic intake are promising strategies to keep a healthy gut microbiome and prevent metabolic disarrangements (Clarke, Murphy et al. 2014; Zhang, Yin et al. 2015; Gomes, de Sousa et al. 2017). The goal of the present study is to evaluate the long-term effect of sleep deprivation and poor sleep quality on human gut microbial composition as a potential mediator of the effects of sleep deprivation on metabolic outcomes.

CHAPTER THREE

Methods

Participants

A total of 60 male and female participants 18 to 35 years old, with a BMI between 18.5 to 27.0 kg/m^2 were recruited from Baylor University. Enrollment was open to all ethnicities. Recruitment was achieved by flyer postings on campus, social media, and emails. All eligible subjects were provided informed consent approved by the Institutional Review Board for Human Subjects at Baylor University. All experimental procedures involved in the study conformed to the ethical considerations of the Helsinki Code.

Study Design

The association of habitual sleeping patterns and the composition of gut microbiota were evaluated in a cross-sectional design. After careful explanation of the study proceedings, interested participants underwent meticulous screening (Visit 1). Exclusion criteria included: presence of metabolic (diabetes, CVD, etc.), autoimmune, or sleep disorders; having consumed antibiotics, probiotics or metformin in the last month; having suffered from any diarrheal or gastrointestinal infections in the last month; currently taking laxatives or fiber supplements; currently following a weight loss intervention; being a smoker; being vegetarian, and being pregnant. Participants were initially asked their average sleep time and according to this answer they were collocated into one of four categories: 1) women short sleep, 2) women adequate sleep, 3) men short sleep,

and 4) men adequate sleep. Seven hours was the cutting point between short versus adequate sleep. We recruited 15 participants per group to make up a total of 60 participants.

Participants fulfilling the inclusion/exclusion criteria were scheduled for a second visit which took place within 3 days from Visit 1. In Visit 2 participants answered four questionnaires: a general Medical History questionnaire, the Pittsburgh Sleep Quality Index (PSQI) survey and the Beck Depression and Beck Anxiety Inventories. At the end of visit 2, participants were provided with two stool sample kits (the OMNIgene® gut kit from DNA genotek Inc, Canada and the DNA/RNA Shield kit from Zymo Research, R11009-T) as well as oral and written instructions on how to collect the stool samples. Participants were also provided with a food log to fill out 24hours prior to the collection of the stool sample.

After 3-5 days from Visit 2, participants reported to the laboratory for Visit 3. At this visit, participants came to the laboratory in the morning after a 12h fast, returning their stool sample and 24h food log. Sample tubes were re-labeled and sent to Baylor College of Medicine for their analysis. Procedures during visit 3 consisted on anthropometric measurements, followed by a DXA scan, blood pressure measurements, and blood sampling. At the end of this visit, a SenseWear^{TM} monitor was placed on the non-dominant arm, right above the triceps. Instructions on how to use the monitor and how to charge it were provided to the participants.

Once participants had used the SenseWear® monitor for seven continuous days they returned it back to the lab in Visit 4. After having collected the total of 60 participants (with half of them being female) data were analyzed (Figure 1).

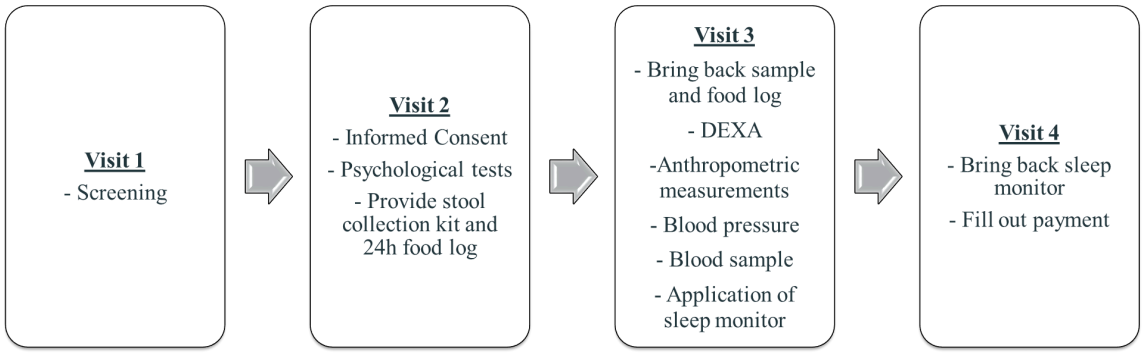

Figure 1. Study Design

Pittsburgh Sleep Quality Index Survey

The PSQI survey assessed the self-reported sleep quality and disturbances over the last month. This survey includes 19 items that evaluate subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, daytime dysfunction, sleep disturbance and use of sleeping medications. The first four items have free-entry responses that assess usual bed and wake times, number of minutes to fall asleep, and hours slept per night, while the remaining items use a 4-point Likert scale. A global sleep quality score was obtained by summing up the scores, with higher scores indicating worse sleep quality (range = 0–21). It has been generally accepted that a PSQI score greater than 5 indicates "poor" sleep quality (Luyster et al., 2015).

The PSQI survey helps us to subjectively analyze sleep quality and to corroborate the results objectively obtained by the sleep monitor. The PSQI survey has shown to have good internal consistency, test-retest reliability and adequate construct and criterion validity for different populations including young adults (Curcio et al., 2013; Popevic et al., 2016; Sohn, Kim, Lee, & Cho, 2012).

Anxiety and Depression Assessment

Anxiety and depression levels were assessed as they both have shown to be correlated with microbiota composition and sleep quality (Kleiman et al., 2015; Moser et al., 2015; Palagini et al., 2014; Viens, De Koninck, Mercier, St-Onge, & Lorrain, 2003; Wigg, Filgueiras, & Gomes Mda, 2014; Nyer et al., 2013). Participants were asked to fill out the Beck Depression and Anxiety Inventories during their second visit with the purpose of isolating the association between sleeping and microbiota parameters from these potential confounding variables.

The Beck anxiety inventory is a self-reported survey containing 21 multiple-choice questions that are used to measure anxiety levels. Participants are asked to indicate to what extent they have been bothered by each specific situation during the past month. The available answers are: not at all, mildly-but it didn't bother me much, moderately-it wasn't pleasant at times, and severely-it bothered me a lot. All columns are added up, to later sum the column totals. If the participant scored from 0-21 the interpretation indicates low levels of anxiety, from 22 to 35 the survey reflects a moderate anxiety, while any number above 36 indicates high anxiety.

The Beck depression inventory is also a self-reported survey with 21 multiple-choice questions that are used to evaluate depression severity. Some of the components evaluate for sadness, feelings about the present and the future, satisfaction, guiltiness, feeling of being punished, self-dislike, self-criticalness, suicidal thoughts or wishes, crying frequency, agitation, loss of interest, indecisiveness, worthlessness, energy loss, sleeping pattern changes, irritability, changes in appetite, concentration difficulty, tiredness or fatigue and loss of interest in sex. Each question can be scored in a scale value of 0 to 3. The interpretation of the survey was reliant upon the sum of the cutoffs values used. A

value between 0 to 13 indicate minimal depression, 14 to 19 mild depression, 20 to 28 moderate depression, 29 to 63 severe depression, and higher scores indicate even more severe depressive symptoms (Beck, Ward, Mendelson, Mock, & Erbaugh, 1961).

Anthropometric Evaluation

Height was measured with a stadiometer to the nearest 0.5 cm. Total body mass was determined by using a calibrated electronic scale with a precision of ± 0.02 kg (Detecto, Webb City, MO). Participants were weighed with minimal clothing and with no shoes, standing on the scale with weight evenly distributed on both feet, arms placed down at sides, standing completely up-right and looking straight ahead. Waist and hip circumference were then measured with a flexible, non-stretchable measuring tape (Baseline Evaluation Instrument, Bolingbrook, IL) at the umbilicus level and at the maximal protuberance of the buttocks, respectively.

Body Composition Testing

Fat mass, fat-free mass, percent body fat, visceral adipose tissue, fat mass to height ratio, and mineral bone density were determined using dual-energy x-ray absorptiometer (Hologic DEXA) after 12h fasting. Subjects were asked to lie in a supine position for approximately six minutes while the scan was performed. No metallic or reflective components were allowed in their clothing the day of the DEXA assessment. Subjects were exposed to a low dosage radiation of approximately 1.5 mR. All scans were performed after a fasting period of 12h and were analyzed by the same Hologic-certified member of the research team.

Metabolic Syndrome Risk Factors

A risk factor for metabolic syndrome was established when one of the following ATPIII criteria was met: fasting glucose >100 mg/dl, triglycerides >150mg/dl, HDL cholesterol <40 mg/dl in men and <50 mg/dl in women, SBP >130 mmHg, DBP >85 mmHg, or abdominal obesity >88cm in women and >102cm in men (Huang 2009).

Blood Pressure Assessment

Blood pressure was assessed by auscultation of the brachial artery using a mercurial sphygmomanometer following standard clinical procedures. The first measurement was taken after having the participant resting for 5 minutes while the second measurement was taken after 5 minutes of the first one. The average of both measurements was recorded for SBP and DBP.

Blood Sample

Participants donated approximately 20 milliliters of venous blood in a single time blood draw using standard phlebotomy procedures. Study personnel wore personal protective clothing (gloves, lab coats, etc.) while drawing blood and handling blood samples. While subjects were seated in a phlebotomy chair, a tourniquet was applied tight enough without causing discomfort. After application of the tourniquet, alcohol pads were used to clean the site. Participants were instructed to make a fist several times in order to maximize venous engorgement. Twenty-one gauge syringes or butterflies were used to puncture the vein by holding them in the dominant hand, entering the skin at a 30-degree angle and in the direction of the vein. After entering the vein the angle was decreased until it was nearly parallel to the skin. The venipuncture site was stabilized by

applying counter tension against the skin just below the entry site using the thumb of the non-dominant hand and carefully pulling the skin toward the wrist. After the blood sample had been collected, the tourniquet was released and a band-aid was then placed to protect the sample site. Needles were disposed in a sharps container while alcohol wipes and gauzes were discarded in an appropriately-labeled biohazard waste receptacle. The blood collection tubes were centrifuged at 3,500 rpm for 20 min. The serum obtained was transferred into 3 labeled microtubes, to be later stored at -80°C (2 microtubes) or transported to the Clinical Pathology Laboratory (1 microtube) for posterior analysis. Clinical Pathology Laboratory performed the analysis of glucose and lipid profile for each participant.

24h Food Log

Participants filled out a 24h food log evaluating the food consumption of the day prior to the stool sample collection. Participants were instructed on how to properly fill out this form during visit 2. Food log included columns for food (including brand or restaurant), cooking method, and amount (in grams or household measure). Participants were encouraged to specify the food ingredients of each of their meals. Energy intake, macronutrient composition, fiber content, and fruits and vegetables consumption were assessed by a nutritionist using the ESHA Research Food Processor Nutrition Analysis Software (Salem, OR).

After nutritional analysis, food logs were re-analyzed for food groups categorization according to the Healthy Eating Index (HEI) (https://epi.grants.cancer.gov/hei/calculating-hei-scores.html). The HEI is a dietary index developed by the United States Department of Agriculture (USDA) as a means to assess

dietary compliance to U.S. Dietary Guidelines. HEI was first developed in 1995, and has been updated every 5 years by collaborative efforts of the USDA, the Center for Nutrition Policy and Promotion, and the National Cancer Institute (Guenther et al. 2013). When compared to the Mediterranean Diet Score and the Healthy Food Diversity Index, the HEI was the best dietary summary to capture gut microbiota variance attributable to habitual diet (Miller et al. 2016). HEI has shown to have the highest number of associations with microbial taxa and explain the greatest variance in diversity (Bowyer et al. 2018). The HEI classifies food into several categories including fruits (fruits and juices), whole fruits (intact fruits, not juices), vegetables, greens and beans (green vegetables, beans, peas, and soy products), whole grains, dairy (milk, yogurt, cheese, and fortified soy beverages), protein sources, seafood and plant proteins, and refined grains. The HEI number of food groups represents the number of group categories that each participant consumed.

Stool Sample

Participants were provided with two stool sample kits (the OMNIgene® gut kit from DNA Genotek Inc, Canada and the DNA/RNA Shield kit from Zymo Research, R11009-T) and were instructed orally and in written form how to collect the stool samples. Stool samples were collected by each participant at their home, between visit 2 and 3, preferentially during the morning. According to manufacturer instructions, the OMNIgene gut kit keeps the sample stable at ambient temperature for up to 60 days (16S rRNA analysis) while the DNA/RNA Shield tube keeps the sample stable at ambient temperature for up to 1 month (for future RNA analysis). The tubes were labeled with an ID number for tracking purposes. Once participants had collected the samples and had

brought the tubes back to the lab, staff members sent them to Dr. Petrosino's Alkek Center for Metagenomics and Microbiome Research Lab, at Baylor College of Medicine, Houston, Texas.

Baylor College of Medicine analyzed the stool samples using high throughput sequencing of the 16S rRNA gene in three batches, the first batch contained 30 samples, whereas the second and third batch contained 15 samples. 16S rRNA gene compositional analysis provides a summary of the composition and structure of the bacterial component of the microbiome. Genomic bacterial DNA extraction methods were optimized to maximize the yield of bacterial DNA while keeping background amplification to a minimum. 16S rRNA gene sequencing methods were adapted from the methods developed for the Earth Microbiome Project and NIH-Human Microbiome Project (Caporaso et al. 2012; Human Microbiome Project 2012b; Human Microbiome Project 2012a). Briefly, bacterial genomic DNA was extracted using MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories). The 16S rDNA V4 region was amplified by PCR and sequenced in the MiSeq platform (Illumina) using the 2x250 bp paired-end protocol yielding pair-end reads that overlap almost completely. The primers used for amplification contain adapters for MiSeq sequencing and single-index barcodes so that the PCR products may be pooled and sequenced directly (3), targeting at least 10,000 reads per sample.

CMMR 16S (variable region 4) rRNA gene pipeline data incorporates phylogenetic and alignment based approaches to maximize data resolution. The read pairs are demultiplexed based on unique molecular barcodes added via PCR during library generation, then merged using USEARCH v7.0.1090 (Edgar 2010). The subsequent

analysis steps of the pipeline leverage custom analytic packages developed at the Alkek Center for Metagenomics and Microbiome Research (CMMR) at Baylor College of Medicine to produce summary statistics and quality control measurements for each sequencing run, as well as multi-run reports and data-merging capabilities for validating built-in controls and characterizing microbial communities across large numbers of samples or sample groups. 16Sv4 rDNA sequences are clustered into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using the UPARSE algorithm (Edgar 2013). OTUs are subsequently mapped to an optimized version of the SILVA Database (Quast et al. 2013) containing only sequences from the v4 region of the 16S rRNA gene to determine taxonomies. Abundances are recovered by mapping the demultiplexed reads to the UPARSE OTUs.

Gene sequence reads are clustered based on their phylogenetic similarity; these clusters receive the name of Operational Taxonomic Unit (OTU)(He et al. 2015). Number of OTUs represents microbial richness, which is the number of different species in a habitat/sample without taking into consideration their abundance (Edgar 2017). Fisher index describes the relationship between the number of species and the number of individuals of the corresponding species by using logarithmic distribution (Jumpponen et al. 2009). Fisher index is not influenced by the sample size and is less affected by the abundance of the most common species (Zhu et al. 2017). Shannon and Simpson indexes are measures of alpha diversity that take into consideration microbial richness and abundance. While Shannon index is more sensitive to richness, Simpson index is more subject to change due to evenness (Kim et al. 2017a). In addition, Shannon index is more sensitive to changes caused by rare OTU, whereas Simpson index is more sensitive to

changes by dominant OTU (Hill et al. 2003). Inverse Simpson was created with the purpose of providing an easier interpretation to Simpson's diversity index. It is calculated as $1/\lambda$ and it estimates the probability that two random reads from a particular sample come from different taxa. A higher value represents greater diversity (Zhu, Wang et al. 2017).

Objective Assessment of Sleep Time and Physical Activity

Although polysomnography has been considered as the gold standard method to measure sleeping time and quality, requiring participants to sleep in the lab setting can alter their sleeping patterns. New portable technologies have been created/modified to measure sleeping parameters. One of such technologies is the SenseWear^{TM} monitor (BodyMedia Inc., Pittsburgh, PA, USA), which was utilized in the present study to objectively measure sleeping time, physical activity duration, energy expenditure, and number of steps per day. SenseWear^{TM} monitor has been validated against polysomnography showing high reliability (Soric et al. 2013; Roane et al. 2015).

Biometric information including date of birth, gender, height, weight, and handedness of the participant were introduced in the equipment before providing the participants with the armband. SenseWear^{TM} was worn on the non-dominant upper arm, over the triceps muscle. Participants were instructed to wear the armband for 7 continuous days removing it only to shower and to charge the monitor according to manufacturer guidelines. Compliance was directly measured, as skin contact is needed in order for the device to collect data. Adherence time is expressed as the average time per day at which each monitor was used out of 24h. A day was considered valid if the monitor was worn at least 22h; duration that was determined according to estimated non-

wear time spent on personal grooming (ie, bath, shower) and required charging time. Once the participants concluded the study, SenseWear™ data were downloaded into the computer using the SenseWear™ 7.0 Professional Software to determine sleeping parameters (2012; Pittsburg, PA). Sleep variables include: average weekly time in bed, standard deviation of sleeping time, average weekdays sleeping time, and average weekends sleeping time. Physical activity variables comprise: average METs per day, sedentary to light (0-3 METs), moderate (>3 to 6 METs), and vigorous physical activity (>6 METs), average time spent in moderate to vigorous physical activity (MVPA) (>3 METs), average time spent in MVPA including only bouts above 10 min (>3 METs), average energy expenditure, and average number of steps per day. Days were divided from noon to noon for analysis involving sleeping variables, and from midnight to midnight for analysis involving physical activity.

Statistical Analyses

An a priori sample size of 40 to 60 participants (~20 to 30 per group) was estimated in order to detect significant changes in the gut microbial composition. According to G-Power 3.1 a sample size of 44 participants was needed in order to detect significant correlations with an effect size $\geq$ 0.4, whereas a sample of at least 26 participants was needed in order to detect a significant correlation with an effect size $\geq$ 0.5. Input variables were 2 tails, alpha error of 0.5, and power of 0.80. According to these numbers the analysis of both the entire, as well as, gender-stratified population will allow us to detect moderate to strong correlations.

After being divided by gender, all variables were checked for normality by using Shapiro-Wilk tests, histograms, and QQ plots. Gender differences were analyzed by

independent groups T-tests. Relationship between variables by Pearson correlations and comparison between extreme sleep groups through Univariate General Linear Regression.

Multivariable linear regression was then used to determine predictors of alpha diversity in this population. A total of six models were developed, three of them to explain Shannon index, while the other three were created to explain Observed OTU. From each of these sets of 3 models, one model utilized the entire population while the other two used gender-specific data as sex seems to be a strong factor controlling microbiome structure (Org et al. 2016). After taking into consideration variables that have previously shown to strongly affect gut microbiome, such as Gender (2 levels), Ethnicity group (4 levels), and Batch number (3 levels), subgroups within the contingency table demonstrated underrepresentation. In order to have enough participants (>5 per category) into each contingency table subgroup, ethnicity was divided into two categories, white (62% of participants) and non-white (38%). Additionally, batch number was reduced into two instead of three categories as no statistical differences in four out of the five alpha diversity measures were found between batch 2 (n=15) and 3 (n=15); however, significant differences in these four alpha diversity measures were found between batch 1 (n=30) and 2+3 (n= 30). Therefore, we decided to merge sets 2 and 3, creating only 2 batch groups. The creation of dichotomous variables in our three categorical variables decreased the number of groups in the contingency table from 24 to 8, providing sufficient representation of participants for each subgroup.

Once the strongest predictors had been identified, they were used as covariates in Hierarchical Linear Regression models. This type of analysis is used to evaluate the

ability of our variable of interest (sleep duration) to explain variance in our dependent variable (alpha diversity) after accounting for all other variables that showed to be significant in multivariable linear regression (gender, ethnicity, batch number, whole fruits consumption, HEI, anxiety, and BMI).

All analyses involving the microbiome were corrected for batch effect. Strength of correlations was considered to be small if ≤ 0.4, moderate if > 0.4 and ≤ 0.6 or large if >0.6. A significant value of 0.05 was adopted throughout. SPSS 11.0 software was used to conduct all statistical analyses.

CHAPTER FOUR

Results

Demographics

A total of 114 participants underwent screening with 60 satisfying inclusion criteria and completing all aspects of the study. Five participants were excluded from statistical analysis, because they did not satisfy the minimum time (23h per day) required for wearing the SenseWear monitor, to guarantee their monitor data would accurately represent their normal physical activity and sleep patterns. In the remaining 55 subjects, the average wearing time of the monitors was 23h 20 min ± 12 min/day. Ethnicity stratified by gender is shown in Table 1. There were no differences in ethnicity between gender. Among participants, 49% were females and 62% classified themselves as being White. Table 1 shows the mean and standard deviation of the following characteristics: anthropometrics, psychological, body composition, diet, sleep, physical activity, blood pressure, blood markers, metabolic syndrome risk, and alpha diversity. All participants ranged from 18-25, with an average of 20.8 years (SD= 1.7), and no significant age differences between genders. Several anthropometric differences were observed between gender. Male participants were significantly taller, heavier, and had a larger waist circumference and BMI in comparison to their female counterparts. No significant in the psychological variables: anxiety and depression were observed between genders. Body composition showed several significant differences by gender.

Table 1. Distribution of variables by gender.

Variables	Total (n=55)		Female (n=27)		Male (n=28)		*p*
	Average	SD	Average	SD	Average	SD	
Ethnicity							
White	34		17		17		0.960
Hispanic	9		4		5		
Asian	7		3		4		
Others	5		3		1		
Age							
Years	20.84	1.72	20.6	1.8	21.1	1.6	0.238
Anthropometrics							
Height (m)	1.7	0.1	1.7	0.1	1.8	0.1	0.000***
Weight (kg)	158.0	28.2	138.2	17.3	177.0	23.3	0.000***
Waist Circumference (cm)	79.4	8.6	75.3	8.1	83.4	7.1	0.001***
Hip Circumference (cm)	98.5	6.9	97.5	6.9	99.5	6.8	0.297
BMI (kg/m2)	23.8	2.4	23.0	2.2	24.7	2.3	0.007**
Psychological variables							
Anxiety (Beck Inventory)	8.93	7.54	10.26	8.59	7.64	6.26	0.201
Depression (Beck Inventory)	6.44	5.31	6.96	5.63	5.93	5.03	0.475
Body Composition							
Fat Mass (gr)	16164.8	4785.6	18162.9	4788.2	14238.0	3985.3	0.002**
Lean Mass (gr)	51795.0	12794.	41388.7	5294.1	61829.6	9364.4	0.000***
Percentage of Fat (%)	23.56	7.52	29.20	5.54	18.12	4.59	0.000***
Fat Mass to Height (kg/m2)	5.51	1.82	6.64	1.62	4.41	1.26	0.000***
VAT area (cm2)	45.00	18.28	39.15	20.37	50.64	14.18	0.018*
Bone Mineral Density (gr/cm2)	1.13	0.12	1.07	0.08	1.19	0.11	0.000***
Diet							
Calories (kcal)	2215.83	767.48	1857.49	615.58	2536.19	781.60	0.001**
Corrected Carbohydrates[1] (gr)	116.97	24.65	121.22	23.45	112.87	25.50	0.212
Corrected Fiber[1] (grams)	9.53	4.73	9.72	5.01	9.35	4.54	0.776
Corrected Protein[1] (grams)	47.72	15.98	45.21	15.47	50.14	16.37	0.257
Protein per Kg[1] (g/kg)	0.68	0.25	0.73	0.28	0.63	0.22	0.147
Corrected Fat[1] (grams)	39.75	11.75	36.13	10.01	43.24	12.40	0.023*
Fruits & Veg. [1] (portions/day)	2.12	2.00	1.92	2.05	2.31	1.96	0.467
HEI Food Groups	5.27	1.73	5.56	1.83	5.00	1.61	0.236
Blood Pressure							
Systolic BP (mmHg)	107.60	10.26	102.11	8.89	112.89	8.67	0.000***
Diastolic BP (mmHg)	71.65	7.47	70.15	6.40	73.11	8.23	0.144
Metabolic Syndrome Risk							
According to ATPIII Criteria	0.51	0.69	0.48	0.80	0.54	0.58	0.774

Continuation

Variables	Total (n=55)		Female (n=27)		Male (n=28)		*p*
	Average	SD	Average	SD	Average	SD	
Physical Activity							
Average METs	1.60	0.24	1.47	0.18	1.73	0.22	0.000
Sedentary/Light PA (0 to < 3)	17:26	4:49	18:20	4:24	17:36	4:07	0.321
Moderate PA (≥ 3 to < 6 METs)	2:14	1:23	1:36	0:46	2:49	1:34	0.000***
Vigorous PA (≥6 METs)	0:10	0:14	0:07	0:11	0:14	0:15	0.030*
MVPA (≥ 3 METs)	2:32	1:23	1:48	0:47	3:15	1:28	0.000***
MVPA bout >10m (≥ 3 METs)	1:47	1:22	1:04	0:43	2:28	1:28	0.000***
Number of Steps	8919.8	3491.3	8069.1	2898.4	9740.0	3855.8	0.076
Energy Expenditure (Kcal)	2703.9	720.8	2138.6	238.9	3249.0	597.2	0.000***
Sedentary with sleep (h:min)	17:10	1:54	17:47	1:38	16:32	1:58	0.017*
Sedentary no sleep (h:min)	10:17	1:47	10:29	1:34	10:04	1:59	0.396
Blood Markers							
Glucose (mg/dl)	95.62	6.64	92.33	4.64	98.79	6.8	0.000***
Triglycerides (mg/dl)	79.04	31.45	85.22	38.78	73.07	21.32	0.154
Cholesterol (mg/dl)	160.25	27.03	168.59	31.61	152.21	19.04	0.023*
HDL (mg/dl)	58.29	15.69	64.00	16.70	52.79	12.63	0.007**
LDL (mg/dl)	86.07	20.83	87.48	24.78	84.71	16.51	0.627
LDL to HDL ratio	1.57	0.53	1.45	0.54	1.69	0.49	0.092
Alpha Diversity							
Observed OTU	81.22	25.89	84.59	28.49	77.96	23.16	0.347
Shannon	2.54	0.68	2.68	0.74	2.40	0.60	0.132
Simpson	0.80	0.16	0.83	0.17	0.78	0.16	0.278
Inv Simpson	8.42	5.54	9.95	6.03	6.94	4.67	0.042*
Fisher	14.13	5.46	14.87	6.08	13.43	4.79	0.331
Sleep							
Weekly Average Sleep (h:min)	6:52	0:56	7:16	0:57	6:28	0:44	0.001***
SD of Sleep (h:min)	1:18	0:17	1:22	0:18	0:45	0:17	0.417
Weekdays Sleep (h:min)	6:44	1:04	7:14	1:04	6:15	0:48	0.000***
Weekend Sleep (h:min)	7:07	1:13	7:18	1:12	6:56	1:14	0.279
Sleep Quality (PSQI Score)	5.04	2.33	4.63	1.98	5.44	2.61	0.202

Carbohydrates, Protein, Fat and Fiber consumption were corrected per every 1,000 of consumed calories.
P values represent comparison between genders.
* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
*** Correlation is significant at the 0.001 level (2-tailed).

Female participants had significantly higher fat mass, fat mass percentage, fat mass to height ratio, and visceral adipose tissue in comparison to male participants, whereas male participants had significantly higher lean mass and bone mineral density. Dietary variables were consistent between genders with only total caloric intake and corrected fat showing to be significantly higher in male participants. The physical activity variables:

average METs, moderate physical activity (≥3 but <6 METs), vigorous physical activity (≥6 METs), MVPA (≥3 METs), MVPA time including only bouts longer than 10 min, and energy expenditure were significantly higher in male participants. Sedentary time was significantly higher in female participants only when including sleep time but not when sleep time was excluded. SBP was significantly higher in male participants, with DBP showing no significant gender-differences. Glucose was significantly higher in male participants, whereas total cholesterol and HDL cholesterol were significantly higher in female participants. No significant differences were observed in metabolic syndrome risk. Gender-differences of gut microbiota and sleep variables will be discussed in their specific subsections.

Dietary Variables and their Association to Gut Microbiota

Female participants had significantly higher Inverse Simpson in comparison to their male counterparts (p= 0.042). No other significant differences between genders were observed among the other alpha diversity indexes (Table 1).

The average amount of calories consumed was 2,215.83 ± 767.48 kcal with a significantly higher caloric intake consumed by male participants (p= .001). The average amounts of corrected carbohydrates, protein and fat consumed were 116.97 ± 24.65, 47.72 ± 15.98, 39.75 ± 11.75 (per 1,000 kcal), respectively. Only fat consumption showed to be significantly higher in men in comparison to women. The average corrected fiber consumption was 9.53 ± 4.73 gr with no significant differences by gender. The average fruits and vegetables consumption was 2.12 ± 2.00 portions with no significant differences between genders (Table 1).

Gender-stratified correlational analysis between nutrition variables and alpha diversity indexes showed no significant associations among females, although a non-significant positive correlation between fruits and vegetables consumption and Inverse Simpson was observed (r= .363, p= .069). Whereas, in male participants, a significant positive correlation between fruits and vegetables consumption and Observed OTU (r= .472, p= .013), as well as Fisher index (r=.457, p= .017) was noted. The rest of the dietary variables (calories consumed, and corrected carbohydrates, fiber, protein, and fat) did not show any significant correlation with alpha diversity measures (Table 2).

After performing the initial analysis to evaluate nutritional value, food logs were assessed for food variety according to the HEI. In female participants, consumption of fruits and whole fruits was positively correlated with Inverse Simpson (r= .397, p= .045; r= .507, p= .008), whereas in male participants fruits and whole fruits were positively correlated with number of OTUs (r= .420, p= .029; r= .533, p= .004) and Fisher index (r= .425, p= .027; r= .543, p= .003). Diary and refined grains showed significant positive correlations with number of OTUs (r= .478, p= .012; r= .443, p= .021) and Fisher index (r= .492, p=.009; r= .455, p= .017, respectively) in male participants. The number of food groups according to the HEI was also positively correlated with Observed OTUs (r= .561, p= .002), Shannon index (r= .534, p= .004), Simpson (r= .421, p= .029), and Fisher index (r= .547, p= .003), representing a positive correlation between food variety and alpha diversity/richness. The only negative correlation observed was between "seafood and plant protein" consumption and Shannon as well as Simpson indexes (r= -.404, p= .041; r= -.397, p= .045) in female participants. The group "seafood and plant protein" includes

seafood, nuts, and seeds as well as soy products. The rest of the variables did not show any significant correlation with alpha diversity measures (Tables 3 and 4).

Anthropometric and Body Composition Variables and their Association with Gut Microbiota

Average height and weight of the participants were 1.7 ± 0.1 m and 158.0 ± 28.2 kg, while their average waist circumference and hip circumference were 79.4 ± 8.6 cm and 98.5 ± 6.9 cm, respectively. With exemption of hip circumference, the rest of the variables were significantly higher in men ($p < .001$). The average BMI was 23.8 ± 2.4 kg/m^2 being also significantly higher in men ($p = .007$) (Table 1). Based on BMI, 58% of the participants were considered as having normal weight with 42% considered as overweight. Female participants had significantly higher fat mass ($p = .002$), fat mass percentage ($p < .001$), fat mass to height ratio ($p < .001$), and visceral adipose tissue ($p = .018$), whereas male participants had significantly higher lean mass ($p < .001$) and mineral bone density ($p < .001$). Based on fat mass to height ratio (fat mass index) 76.4% of the participants were considered as having normal fat mass with 11% classified as having excess fat. Mild and moderate fat deficit were also detected with a frequency of 9% and 3.6%, respectively.

Microbial diversity variables did not show any significant direct correlation with gender-stratified body composition parameters such as: fat mass, lean mass, body fat percentage, fat mass to height ratio, visceral adipose tissue, and bone mineral density (Table 5).

Table 2. Correlation between Dietary and Alpha Diversity Variables

Variable	Pearson Values	Observed OTU	Shannon	Simpson	Inverse Simpson	Fisher
		Female (n= 27)				
Calories Consumed	Correlation	-.219	-.010	.075	.008	-.212
	Sig. (2-tailed)	.283	.962	.716	.967	.299
Corrected Carbohydrates[1]	Correlation	-.233	-.240	-.205	-.234	-.235
	Sig. (2-tailed)	.252	.238	.315	.251	.248
Corrected Fiber[1]	Correlation	-.185	.024	-.005	.155	-.194
	Sig. (2-tailed)	.365	.906	.981	.451	.341
Corrected Protein[1]	Correlation	.065	-.210	-.296	-.053	.067
	Sig. (2-tailed)	.754	.304	.142	.796	.744
Corrected Fat[1]	Correlation	.191	.285	.242	.229	.195
	Sig. (2-tailed)	.351	.159	.234	.259	.339
Fruits and Vegetables	Correlation	.173	.294	.243	.363	.172
	Sig. (2-tailed)	.399	.145	.232	.069	.401
		Male (n= 28)				
Calories Consumed	Correlation	.212	.067	.049	-.117	.200
	Sig. (2-tailed)	.288	.739	.810	.560	.318
Corrected Carbohydrates[1]	Correlation	.093	-.178	-.260	-.113	.111
	Sig. (2-tailed)	.645	.375	.190	.576	.581
Corrected Fiber[1]	Correlation	.031	.272	.255	.280	.022
	Sig. (2-tailed)	.879	.169	.200	.158	.912
Corrected Protein[1]	Correlation	.032	.158	.197	.059	.022
	Sig. (2-tailed)	.875	.430	.324	.770	.912
Corrected Fat[1]	Correlation	-.248	.046	.180	.005	-.260
	Sig. (2-tailed)	.211	.819	.368	.980	.189
Fruits and Vegetables	Correlation	.472*	.340	.241	.245	.457*
	Sig. (2-tailed)	.013	.082	.226	.217	.017

[1] Carbohydrates, Protein, Fat and Fiber consumption were corrected per every 1,000 of consumed calories
* Correlation is significant at the 0.05 level (2-tailed).

Table 3. Correlation between Healthy Eating Index Food Groups and Alpha Diversity in Female Participants

Variables	Pearson Values	Observed OTU	Shannon	Simpson	Inverse Simpson	Fisher
Fruits	Correlation	.228	.321	.255	.397*	.230
	Sig. (2-tailed)	.263	.110	.208	.045	.258
Whole fruits	Correlation	.246	.352	.244	.507**	.253
	Sig. (2-tailed)	.225	.077	.230	.008	.213
Vegetables	Correlation	.080	.296	.322	.301	.071
	Sig. (2-tailed)	.698	.142	.108	.135	.730
Greens and beans	Correlation	-.229	.042	.180	-.020	-.236
	Sig. (2-tailed)	.260	.837	.380	.923	.245
Whole grains	Correlation	.020	.201	.226	.171	.000
	Sig. (2-tailed)	.923	.325	.266	.403	1.000
Dairy	Correlation	.027	.022	-.039	-.026	.003
	Sig. (2-tailed)	.895	.914	.852	.899	.989
Protein sources	Correlation	.169	.105	.066	.258	.189
	Sig. (2-tailed)	.408	.609	.750	.204	.354
Seafood and Plant Protein	Correlation	-.302	-.404*	-.397*	-.267	-.312
	Sig. (2-tailed)	.134	.041	.045	.187	.120
Refined grains	Correlation	-.236	-.219	-.141	-.277	-.222
	Sig. (2-tailed)	.245	.283	.492	.171	.276
HEI number of groups	Correlation	.051	.262	.245	.310	.033
	Sig. (2-tailed)	.806	.195	.228	.123	.875

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table 4. Correlation between Healthy Eating Index Food Groups and Alpha Diversity in Male Participants

Variables	Pearson Values	Observed OTU	Shannon	Simpson	Inverse Simpson	Fisher
Fruits	Correlation	.420*	-.078	-.147	-.258	.425*
	Sig. (2-tailed)	.029	.698	.465	.193	.027
Whole fruits	Correlation	.533**	.030	-.035	-.183	.543**
	Sig. (2-tailed)	.004	.883	.862	.360	.003
Vegetables	Correlation	.102	.290	.336	.221	.083
	Sig. (2-tailed)	.613	.142	.087	.269	.679
Greens and beans	Correlation	.203	.341	.346	.236	.183
	Sig. (2-tailed)	.310	.081	.077	.235	.360
Whole grains	Correlation	.165	.235	.206	.166	.157
	Sig. (2-tailed)	.410	.238	.302	.408	.433
Dairy	Correlation	.478*	.066	-.022	-.066	.492**
	Sig. (2-tailed)	.012	.744	.914	.744	.009
Protein sources	Correlation	.070	.117	.208	-.105	.052
	Sig. (2-tailed)	.730	.562	.297	.603	.798
Seafood and Plant Protein	Correlation	.278	.322	.184	.275	.271
	Sig. (2-tailed)	.160	.102	.358	.165	.171
Refined grains	Correlation	.443*	-.025	-.035	-.288	.455*
	Sig. (2-tailed)	.021	.902	.863	.146	.017
HEI groups	Correlation	.562**	.534**	.421*	.346	.547**
	Sig. (2-tailed)	.002	.004	.029	.077	.003

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table 5. Correlation between Body Composition and Alpha Diversity Variables

Variables	Pearson Values	Observed OTU	Shannon	Simpson	Inverse Simpson	Fisher
		Female (n=27)				
Fat Mass (gr)	Correlation	-.074	-.018	.040	-.060	-.069
	Sig. (2-tailed)	.721	.931	.846	.772	.736
Lean Mass (gr)	Correlation	.043	.119	.176	.149	.060
	Sig. (2-tailed)	.836	.564	.388	.468	.772
Percentage Fat Mass (%)	Correlation	-.096	-.051	-.005	-.106	-.100
	Sig. (2-tailed)	.642	.806	.981	.606	.625
Fat Mass to Height	Correlation	-.149	-.128	-.080	-.154	-.149
	Sig. (2-tailed)	.468	.533	.696	.454	.466
VAT area (cm^2)	Correlation	-.004	-.047	-.052	-.013	.003
	Sig. (2-tailed)	.986	.820	.799	.950	.989
BMD (g/cm2)	Correlation	.209	.125	.023	.188	.227
	Sig. (2-tailed)	.305	.542	.912	.357	.265
		Male (n= 28)				
Fat Mass (gr)	Correlation	-.079	-.080	-.105	-.036	-.092
	Sig. (2-tailed)	.695	.691	.604	.858	.648
Lean Mass (gr)	Correlation	.135	-.214	-.222	-.331	.142
	Sig. (2-tailed)	.502	.285	.265	.092	.479
Percentage Fat Mass (%)	Correlation	-.104	.037	.012	.137	-.118
	Sig. (2-tailed)	.605	.856	.951	.495	.557
Fat Mass to Height	Correlation	-.077	-.064	-.118	.038	-.090
	Sig. (2-tailed)	.702	.752	.556	.852	.654
VAT area (cm2)	Correlation	-.195	.026	.047	.068	-.215
	Sig. (2-tailed)	.330	.897	.817	.736	.281
BMD (g/cm2)	Correlation	-.147	-.168	-.113	-.206	-.144
	Sig. (2-tailed)	.464	.403	.575	.302	.474

Physical Activity and its Association to Gut Microbiota

The average time of MVPA was 2:32 ± 1:23h per day, with men performing more physical activity than women (3:15 ± 1:28h versus 1:48 ± 0:47h, respectively, $p < .001$) (Table 1).

All the participants satisfied ACSM recommendations for PA of at least 150 min of MVPA per week (Garber et al. 2011). However, when considering only MVPA bouts of 10 minutes and longer the average daily physical activity decreased to 1:47 ± 1:22h per day, with men continuing to have a significantly higher physical activity level than women (2:28 ± 1:28h versus 1.04 ± 0:43h respectively, $p < .001$). When considering only bouts of 10 min or longer, the number of participants satisfying ACSM recommendations decreased to 83%. The average number of steps was 8,919.81 ± 3,491.33 steps per day, with a tendency to be higher in male participants ($p = .076$). The average energy expenditure was 2,703.96 ± 720.88 kcal, with a significantly higher energy expenditure by males ($p < .001$). The average sedentary time of the entire population including sleep time was 17:10 ± 1:54 h, with female participants having higher sedentary time than male participants (17:47 ±1:38 versus 16:32 ± 1:58, $p = .017$); however, after subtracting sleep, time spent in sedentary activities was no significantly different between genders ($p = 0.396$).

Exercise training has been previously correlated to higher alpha diversity (Monda et al. 2017), however, in our cross-sectional study physical activity variables did not correlate to alpha diversity measures (Table 6).

Table 6. Correlation between Physical Activity and Alpha Diversity Variables

Variables	Pearson Values	Observed OTU	Shannon	Simpson	Inverse Simpson	Fisher
		Female (n= 27)				
Energy Expenditure (Kcal)	Correlation	-.171	.139	.288	-.016	-.167
	Sig. (2-tailed)	.404	.498	.154	.938	.414
Total PA (hours)	Correlation	-.218	-.070	-.024	-.189	-.231
	Sig. (2-tailed)	.284	.734	.907	.355	.257
PA (including only bouts >10min)	Correlation	-.244	-.044	.005	-.130	-.256
	Sig. (2-tailed)	.229	.832	.981	.526	.208
Number of Steps	Correlation	-.191	-.019	.020	-.123	-.193
	Sig. (2-tailed)	.350	.926	.922	.551	.345
Weekly Sedentary Time (including sleep)	Correlation	.194	.052	.065	.063	.200
	Sig. (2-tailed)	.354	.804	.757	.765	.337
Weekly Sedentary Time (not including sleep)	Correlation	.223	.187	.237	.157	.223
	Sig. (2-tailed)	.285	.371	.253	.455	.285
		Male (n= 28)				
Energy Expenditure (Kcal)	Correlation	.103	-.140	-.121	-.268	-.121
	Sig. (2-tailed)	.608	.486	.548	.176	.548
Total PA (hours)	Correlation	.090	-.036	.012	-.147	.012
	Sig. (2-tailed)	.656	.860	.953	.464	.953
PA (including only bouts >10min)	Correlation	.172	-.024	.012	-.159	.012
	Sig. (2-tailed)	.391	.903	.951	.428	.951
Number of Steps	Correlation	.084	.043	.071	-.106	.071
	Sig. (2-tailed)	.676	.830	.727	.599	.727
Weekly Sedentary Time (including sleep)	Correlation	-.011	.034	.022	.105	-.010
	Sig. (2-tailed)	.960	.872	.917	.618	.964
Weekly Sedentary Time (not including sleep)	Correlation	.121	.045	-.001	.093	.122
	Sig. (2-tailed)	.564	.831	.996	.658	.563

Metabolic Syndrome and Cardiovascular Risk Factors and their Association to Gut Microbiota

The average SBP and DBP were 107.60 ± 10.26 mmHg and 71.65 ± 7.47 mmHg, respectively. Only SBP showed a significant difference by gender being higher in males ($p < .001$) (Table 1).

The average values for blood biomarkers were: glucose 95.62 ± 6.64 mg/dl, triglycerides 79.04 ± 31.45 mg/dl, total cholesterol 160.25 ± 27.03 mg/dl, HDL cholesterol 58.29 ±15.69 mg/dl, LDL cholesterol 86.07 ± 20.83 mg/dl, and LDL/HDL ratio 1.5 mg/dl. While glucose was significantly higher in males ($p < .001$), total cholesterol and HDL cholesterol were significantly higher in females ($p = .023$ and $p = .007$ respectively) (Table 1). Most of the participants did not have any metabolic syndrome risk (58%), 34.5% had one risk, 5.5% two risks, and only 1.8% had three risk factors. The average metabolic syndrome risk score was 0.51 with no significant differences between genders.

Correlations between alpha diversity indicators and metabolic syndrome risk factors were not significantly correlated in female participants. However, male participants had several significant correlations. Specifically, LDL cholesterol and LDL to HDL ratio were positively correlated with Inverse Simpson ($r = .435$, $p = .023$; $r = .390$, $p = .044$, respectively). A significant positive correlation between DBP and Fisher index was also observed in this population ($r = .444$, $p = .020$). On the other hand, BMI was negatively correlated with Simpson index ($r = -.414$, $p = .032$). Total cholesterol, triglycerides, HDL cholesterol, glucose, and SBP did not show any significant correlation with alpha diversity measures in male participants (Table 7 and 8).

Table 7. Correlation between Metabolic Syndrome and Cardiovascular Risk Factors with Alpha Diversity Variables in Female Participants

Variables	Pearson Values	Observed OTU	Shannon	Simpson	Inverse Simpson	Fisher
Cholesterol	Correlation	.026	.112	.071	.164	.028
	Sig. (2-tailed)	.899	.586	.732	.423	.890
TAG	Correlation	.002	.157	.206	.100	-.002
	Sig. (2-tailed)	.992	.444	.313	.628	.991
HDL	Correlation	-.008	.100	.022	.155	.008
	Sig. (2-tailed)	.969	.627	.915	.450	.971
LDL	Correlation	.040	.023	.008	.074	.033
	Sig. (2-tailed)	.848	.910	.970	.720	.873
LDL to HDL Ratio	Correlation	-.016	-.095	-.034	-.088	-.030
	Sig. (2-tailed)	.939	.643	.868	.669	.886
Glucose	Correlation	-.039	.044	.140	-.036	-.049
	Sig. (2-tailed)	.850	.833	.495	.860	.812
SBP	Correlation	-.009	-.063	-.024	-.078	.002
	Sig. (2-tailed)	.964	.760	.907	.705	.994
DBP	Correlation	-.049	-.240	-.284	-.070	-.032
	Sig. (2-tailed)	.811	.237	.160	.735	.875
BMI (kg/m^2)	Correlation	-.205	-.196	-.143	-.148	-.198
	Sig. (2-tailed)	.315	.338	.487	.470	.333
Waist C. (cm)	Correlation	-.142	-.048	.022	-.036	-.128
	Sig. (2-tailed)	.488	.816	.914	.862	.532

Table 8. Correlation between Metabolic Syndrome and Cardiovascular Risk Factors with Alpha Diversity Variables in Male Participants

Variables	Pearson Values	Observed OTU	Shannon	Simpson	Inverse Simpson	Fisher
Cholesterol	Correlation	-.004	.195	.168	.320	.007
	Sig. (2-tailed)	.982	.331	.403	.103	.972
TAG	Correlation	-.072	.018	.028	.050	-.064
	Sig. (2-tailed)	.720	.927	.891	.804	.751
HDL	Correlation	-.144	-.069	.069	-.108	-.142
	Sig. (2-tailed)	.475	.731	.732	.591	.481
LDL	Correlation	.128	.272	.135	.435*	.138
	Sig. (2-tailed)	.525	.171	.502	.023	.494
LDL to HDL Ratio	Correlation	.157	.243	.070	.390*	.161
	Sig. (2-tailed)	.434	.221	.730	.044	.423
Glucose	Correlation	.083	.112	.065	.118	.088
	Sig. (2-tailed)	.679	.577	.747	.559	.663
SBP	Correlation	.328	.174	.069	.049	.317
	Sig. (2-tailed)	.094	.386	.733	.807	.108
DBP	Correlation	.429*	-.142	-.232	-.218	.444*
	Sig. (2-tailed)	.026	.479	.245	.275	.020
BMI (kg/m^2)	Correlation	.030	-.322	-.414*	-.264	.030
	Sig. (2-tailed)	.882	.102	.032	.183	.880
Waist C. (cm)	Correlation	.090	-.115	-.161	-.131	.083
	Sig. (2-tailed)	.657	.569	.424	.515	.679

* Correlation is significant at the 0.05 level (2-tailed).

Anxiety and Depression and their Association to Gut Microbiota

The average anxiety and depression scores in females were 10.26 ± 8.59 and 6.96 ± 5.63, while the average anxiety and depression scores in male participants were 7.64 ± 6.26 and 5.93 ± 5.03, respectively (Table 1). The prevalence of depression (above normal) and anxiety (moderate levels) in our female population were 18.5% and 11%, whereas the prevalence of these factors in our male population were 16.8% and 7%, respectively.

Neither depression nor anxiety scores were associated with alpha diversity in females. However, negative correlations were observed between anxiety and three alpha diversity measures: Shannon index (r= -.552, p= .003), Simpson (r= -.637, p< .000), and Inverse Simpson (r= -.422, p= .028) in male participants (Table 9)(Figures 2, 3 and 4).

Table 9. Correlation between Psychological and Alpha Diversity Variables

Variables	Pearson Values	Observed OTU	Shannon	Simpson	Inverse Simpson	Fisher
		Women (n= 27)				
Depression	Correlation	.118	.029	.056	.025	.132
	Sig. (2-tailed)	.564	.889	.787	.905	.520
Anxiety	Correlation	.055	.085	.118	-.001	.053
	Sig. (2-tailed)	.791	.679	.567	.995	.798
		Men (n= 28)				
Depression	Correlation	-.064	-.173	-.212	-.055	-.074
	Sig. (2-tailed)	.751	.389	.287	.783	.714
Anxiety	Correlation	-.080	-.552**	-.637***	-.422*	-.076
	Sig. (2-tailed)	.692	.003	.000	.028	.705

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
*** Correlation is significant at the 0.001 level (2-tailed).

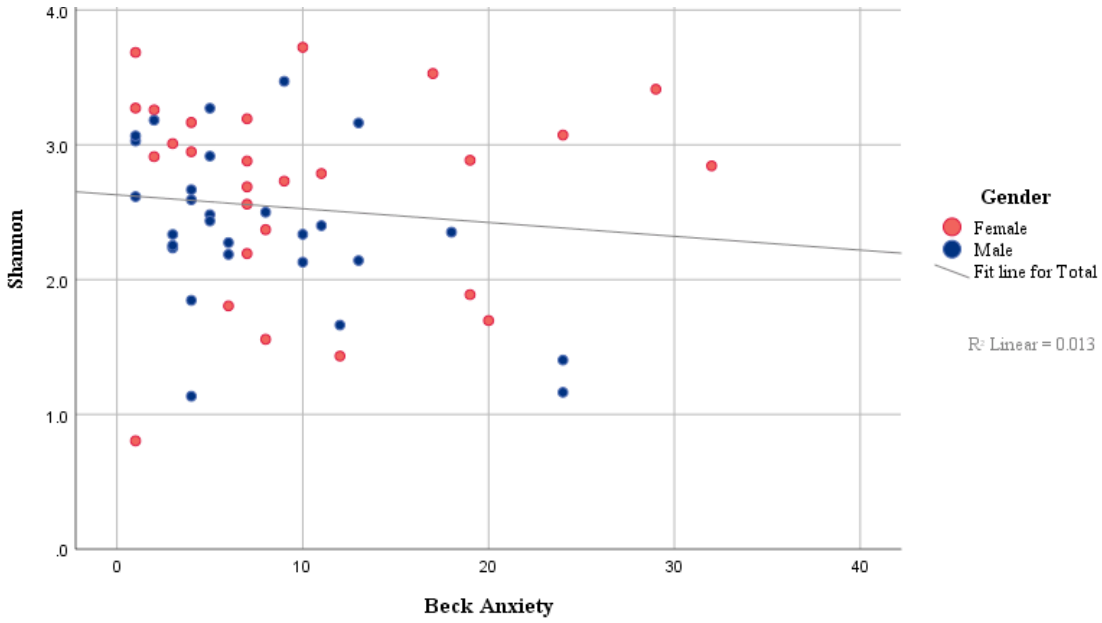

Figure 2. Scatterplot with Fit Line of Shannon by Beck Anxiety by Gender

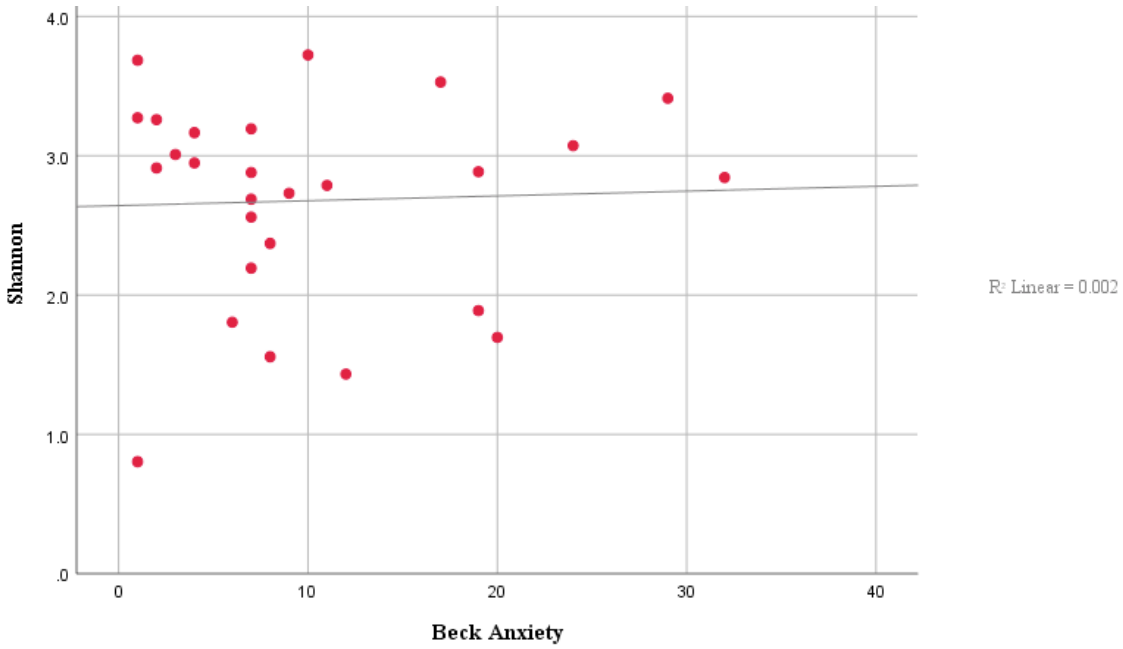

Figure 3. Scatterplot with Fit Line of Shannon by Beck Anxiety in Female Participants

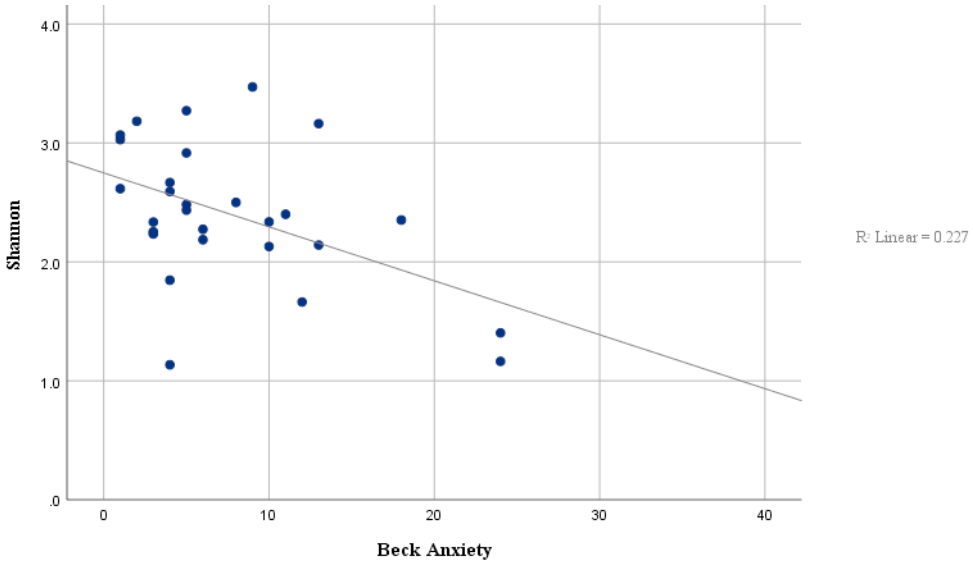

Figure 4. Scatterplot with Fit Line of Shannon by Beck Anxiety in Male Participants

Sleep and its Association to Gut Microbiota

The average weekly sleep time of the participants was 6:52 ± 0:56 h, the average weekdays sleep time was 6:44 ± 1:04 h, while the average weekend sleep time was 7:07 ± 1:13 h. With exception of weekend sleep, both weekly and weekdays sleep were significantly higher in women than men (p= .001 and p< .001, respectively) (Table 1). According to the NSF, 60% of the participants were classified as short sleepers (<7h of sleep), 30% as average sleepers (7-8h), and 10% as long sleepers (>8h). The average sleep quality score according to PSQI was 5.04 ± 2.33 with no significant differences between genders. According to this index, over 54.5% of the selected participants were classified as having poor sleep quality.

Correlation between sleep variables and gut microbiota demonstrated a negative association between weekdays average sleep and Shannon (r= -.422, p= .028) as well as Simpson indexes (r= -.456, p= .017) in female participants. No significant associations were found between alpha diversity and sleep variables in male participants (Table 10).

Prediction Modeling for Alpha Diversity

Shannon index and number of OTUs were chosen as the dependent variables in the six models of multivariable linear regression as both variables were normally distributed and are widely used (Figure 5) (Hill, Walsh et al. 2003). Number of OTUs was significantly correlated with Fisher index as both parameters measure microbial richness. Additionally, both measures resulted in the selection of the same predictors for each of the 6 models. Shannon index was chosen because it significantly correlated with Simpson and Inverse Simpson indexes, as they are all indicators of microbial richness and abundance. However, slight differences in the selected predictors were noted.

Taking into consideration the three models (entire population, males and females) for each alpha diversity measure, Shannon index showed fruits and vegetables, seafood and plant proteins, HEI, BMI, ethnicity, anxiety, and batch number as the main predictors; Simpson index showed BMI, ethnicity, anxiety, and weekdays sleep as the main predictors, as lastly, Inverse Simpson showed whole fruits, refined grains, energy expenditure, LDL concentration, and batch number to be the main predictors (data not shown). Is interesting to note that a batch effect was mainly noticeable in female participants but not in male participants, even though the distribution of genders by batch number did not differ. It is possible that the variables collected in female participants have a higher standard deviation caused by hormonal changes. This situation could prevent from observing significant correlations, thus, leaving batch effect as the only or one of the few strong predictors of alpha diversity in female population.

Table 10. Correlation between Sleep Variables and Alpha Diversity

Variables	Parameter	Observed OTU	Shannon	Simpson	Inverse Simpson	Fisher
		Women (n= 27)				
Sleep time	Correlation	-.144	-.307	-.338	-.209	-.129
	Sig. (2-tailed)	.484	.127	.091	.305	.530
SD of sleep	Correlation	.330	.141	-.014	.263	.337
	Sig. (2-tailed)	.100	.494	.946	.195	.093
Weekdays sleep	Correlation	-.203	-.400*	-.435*	-.278	-.190
	Sig. (2-tailed)	.319	.043	.026	.170	.352
Weekend sleep	Correlation	.185	.174	.149	.143	.189
	Sig. (2-tailed)	.366	.396	.467	.486	.355
Sleep Quality	Correlation	-.027	-.067	-.034	-.228	-.033
	Sig. (2-tailed)	.893	.741	.866	.253	.869
		Men (n= 28)				
Sleep time	Correlation	-.182	-.023	.026	.025	-.185
	Sig. (2-tailed)	.364	.911	.897	.901	.356
SD of sleep	Correlation	-.162	-.236	-.206	-.098	-.152
	Sig. (2-tailed)	.420	.236	.302	.627	.448
Weekdays sleep	Correlation	-.084	.019	.020	.053	-.084
	Sig. (2-tailed)	.676	.924	.923	.792	.677
Weekend sleep	Correlation	-.364	-.057	.069	.024	-.373
	Sig. (2-tailed)	.062	.779	.731	.904	.055
Sleep Quality	Correlation	.012	-.279	-.287	-.278	.005
	Sig. (2-tailed)	.953	.159	.147	.160	.981

* Correlation is significant at the 0.05 level (2-tailed).

Overall, the strongest predictive variables for alpha diversity according to the Shannon index are: variety in the diet (number of HEI food groups), seafood and plant proteins consumption, sleep time, BMI, anxiety, ethnicity, and batch number, whereas the overall strongest predictors for number of OTUs are: whole fruits, sleep time, and batch number (Tables 11 and 12).

To better understand the 3 models predicting alpha diversity through the Shannon index is important to consider its variation range in our population, from .8 to 3.7, with an average of 2.54 ± 0.68.

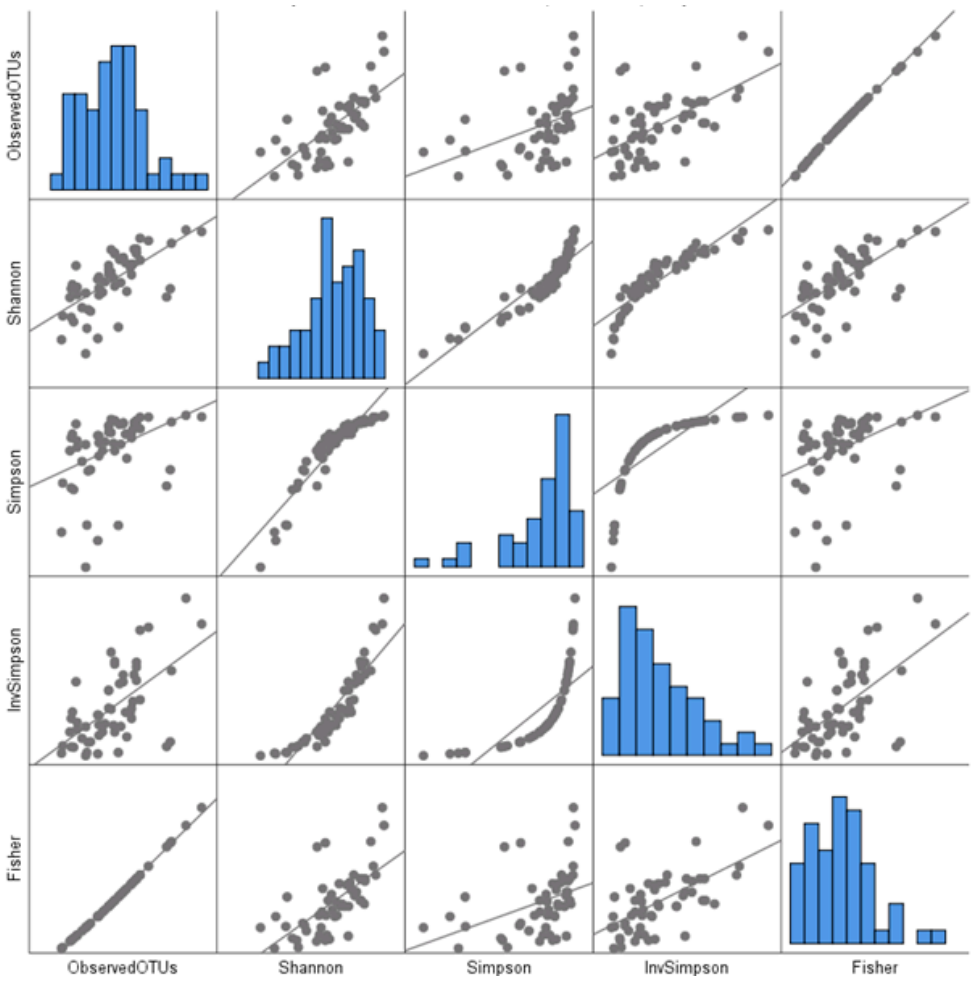

Figure 5. Scatterplot Matrix for Alpha Diversity Variables

Model 1 predicts 25% of the changes in Shannon diversity observed in the total population. The model revealed that BMI, fruits and vegetables consumption, and batch number were the strongest predictors of alpha diversity when considering the total number of participants (n= 55). For every increase in 1 kg/m^2 in the BMI scale, alpha diversity decreased.087 units; for every increase in 1 portion of fruits and vegetables, alpha diversity increased by .100 units, whereas analyzing the sample in the 2^{nd} or 3^{rd} batch increased alpha diversity by .380 units.

Table 11. Multivariable Linear Regression Models using Shannon Index as a Dependent Variable

Variables	Pearson Values	Unstandardized Coefficients		Standardized Coefficients	t	Significance
		B	SE	β		
(1) Total (n= 55)	R^2 .249 (p= .024*)					
	(Constant)	3.840	.847		4.535	.000
	BMI	-.087	.034	-.312	-2.565	.013
	Fruits and Veg.	.100	.041	.293	2.410	.020
	Batch number	.380	.164	.282	2.325	.024
(2) Female (n=27)	R^2 .731 (p= .004**)					
	(Constant)	.499	.481		1.036	.311
	Batch number	.955	.224	.650	4.267	.000
	Seafood_PlantProtein	-.288	.078	-.624	-3.695	.001
	HEI good groups	.226	.072	.505	3.154	.004
(3) Male (n= 28)	R^2 .468 (p= .050*)					
	(Constant)	5.293	.977		5.418	.000
	Anxiety	-.035	.015	-.371	-2.434	.023
	Ethnicity	-.476	.180	-.402	-2.650	.014
	BMI	-.079	.038	-.309	-2.068	.050

* Significant at the 0.05 level
** Significant at the 0.01 level

Table 12. Multivariable Linear Regression Models using Number of OTUs as a Dependent Variable

Variables	Pearson Values	Unstandardized Coefficients		Standardized Coefficients	t	Significance
		B	SE	β		
(4) Total (n= 55)	R^2 .201 (p= .012*)					
	(Constant)	49.2565	10.512		4.686	.000
	Whole Fruits	9.701	3.485	.347	2.783	.007
	Batch number	16.772	6.425	.326	2.610	.012
(5) Female (n= 27)	R^2 .167 (p= .034*)					
	(Constant)	51.886	15.487		3.350	.003
	Batch number	23.239	10.389	.408	2.237	.034
(6) Male (n= 28)	R^2 .467 (p= .012*)					
	(Constant)	67.431	5.558		12.132	.000
	Whole Fruits	12.550	4.665	.467	2.690	.012

* Significant at the 0.05 level

In females, Model 2 predicts 73% of the variation observed in Shannon index in our female population. The strongest predictors in this scenario were: seafood and plant protein, HEI, and batch number. An increase in the consumption of 1 potion of seafood and plant protein, decreased alpha diversity measured through Shannon by .288. By including an additional food group as defined by the HEI (regardless of the source) will increase the Shannon diversity index by .226. Having the sample analyzed in the 2nd or 3rd batch increased alpha diversity by .955 in comparison to batch 1.

In males, Model 3 explained 47% of the variation in alpha diversity. Anxiety, ethnicity, and BMI were the strongest predictors of alpha diversity in this population. An increase of 1 point in the Beck anxiety scale caused a decrease in alpha diversity of .035 units; being non-white decreased alpha diversity by .476 in comparison to being white; and an increase of 1kg/m^2 in BMI decreased alpha diversity by .079 units.

To better understand the three models involving number of OTUs as the dependent variable it is useful to consider the range of observed OTUs in our population, which was 39 to 155, with a mean of 81.22 ± 25.89.

Model 4 explains 20% of the alpha diversity changes observed in our entire population by using whole fruits consumption and batch number. An increase in one portion of fruit consumption increased the number of OTU by 9.7 units. Having the sample analyzed in the 2nd or 3rd batch increased alpha diversity measured through Observed OTU by 16.77 units.

In gender specific models, Model 5 predicts 17% of the changes observed in the number of OTU in our female participants by using only one variable. Having the sample analyzed in the 2nd or 3rd batches caused an increase of 23.3 units in the number of OTUs in comparison to having the sample analyzed in the 1st batch.

In males, Model 6 explains 47% of the changes observed in OTU by using the sole variable of whole fruits consumption. For every increase in the number of whole fruits consumed, an increase of 12.55 units in the number of OTU took place.

Once the strongest predictors had been identified, they were used as covariates in 6 hierarchical regression models. When analyzing the entire population, any of the 5 initial hierarchical regression models (number 1), which controlled for strong confounding factors (gender, ethnicity, batch number, whole fruits consumption, HEI, anxiety, and BMI) showed sleep to be a significant predictor of alpha diversity (Tables 13, 16, 19, 22 and 25). Although the 5 models showed that the combination of our 7 covariates created a model that significantly predicted each of the alpha diversity indexes, the addition of average sleep time creating model 2 did not improve the already existent model 1. The

addition of sleep to the initial models (number 1) only added 0.2 to 1.4% of additional explanatory power to predict each of the 5 alpha diversity measures (Shannon, Simpson, Inverse Simpson, Fisher and Number of OTUs). When analyzing only female participants, any of the initial models containing our 7 covariates (models number 1) showed to be significant to predict any of the 5 alpha diversity variables (Tables 14, 17, 20, 23, and 26). However, when adding sleep to these models, two of them became significant to predict Shannon and Simpson Indexes. The prediction power added by including sleep into models 1 (thus creating models 2) varied from 5% to 14.1%. Contrasting to what we observed in female participants, when analyzing male participants, four out of five initial models containing the 7 covariates showed to have significant strength to predict each of the alpha diversity measures (Tables 15, 18, 21, 24, and 27). Only one model number 1 showed a non-significant tendency to predict Inverse Simpson ($p= .056$). surprisingly, and also in opposite direction to what was observed in female participants, addition of sleep converted all the models in non-significant. The prediction power added by including sleep into models 1 ranged from 0.5 to 1.5%.

When comparing the predictive strength (R^2) of each of our 3 populations across alpha diversity indexes, we noticed that the average predictive power of models 1 and 2 were higher in male participants ($R^2 = 0.51$ and 0.52, respectively) in comparison to female participants ($R^2 = 0.36$ and 0.44) or the entire population ($R^2 = 0.28$ and 0.29). Comparison of adjusted R^2 for models 1 and 2 showed similar results, with models performed in male participants (adjusted $R^2 = 0.37$ and 0.35) presenting higher strength versus those in female participants (adjusted $R^2 = 0.17$ and 0.24) or the entire population (adjusted $R^2 = 0.18$ and 0.17, respectively). When comparing the predictive strength (R^2

and adjusted R^2) of our 5 alpha diversity indexes across our populations, we observed that the models used to predict Simpson index had the highest strength ($R^2 = 0.47$ and adjusted $R^2 = 0.33$), followed by Shannon index ($R^2 = 0.46$ and adjusted $R^2 = 0.32$), Inverse Simpson ($R^2 = 0.37$ and adjusted $R^2 = 0.21$), and lastly both Fisher Index ($R^2 = 0.35$ and adjusted $R^2 = 0.18$) and Number of OTUs ($R^2 = 0.35$ and adjusted $R^2 = 0.18$) (data not shown).

Dietary Variables and their Association to Sleep

Due to the fact that sleep time was significantly different between genders and not uniformly distributed, we decided to separate all sleep-related analyses by gender in order to prevent spurious results.

Caloric intake showed a significant negative correlation with sleep standard deviation in male participants (r= .418, p= .027) whereas fiber intake showed a significant negative correlation with sleep quality in female participants (r= -.412, p=.033); the rest of the gender-stratified dietary variables (corrected fat and protein) did not show any significant correlation with sleep indicators (Table 28).

Analysis of the HEI food groups showed a significant negative correlation between the "greens and beans" group (a combination of dark vegetables, beans, and peas) and weekend sleep time (r= -.424, p= .028), as well as a negative association between "total protein food" and sleep quality in female participants (r= -.417, p= .031). However, in male participants a positive correlation was observed between "green beans" and weekend sleep time (r= .391, p= .040). Also in male participants a significant negative association between sleep time and the group "seafood and plant proteins" was noted (r= -.383, p= .044)(Tables 29 and 30).

Table 13. Hierarchical Linear Regression Model for the Entire Population using Shannon Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	2.459		3.205	
Gender	.369	.275	.424	.316
Ethnicity	-.222	-.159	-.222	-.159
Batch Number	.504	.374	.468	.347
BMI	-.049	-.177	-.052	-.186
Anxiety	-.003	-.028	-.003	-.030
Whole Fruits	-.004	-.005	-.010	-.014
HEI	.125	.320	.117	.298
Sleep Time	-	-	-.001	-.123
R^2	.335		.347	
Adjusted R^2	.237		.233	
Significance	.005		.383	

Table 14. Hierarchical Linear Regression Model for Female Participants using Shannon Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	3.388		6.398	
Ethnicity	-.473	-.316	-.505	-.338
Batch Number	.850	.578	.782	.532
BMI	-.061	-.185	-.085	-.258
Anxiety	.024	.284	.032	.376
Whole Fruits	.286	.389	.357	.485
HEI	-.066	-.148	-.139	-.311
Sleep Time	-	-	-.005	-.373
R^2	.409		.526	
Adjusted R^2	.232		.351	
Significance	.074		.043	

Table 15. Hierarchical Linear Regression Model for Male Participants using Shannon Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	3.486		4.326	-.292
Ethnicity	-.333	-.273	-.357	.256
Batch Number	.345	.295	.300	-.247
BMI	-.064	-.251	-.063	-.344
Anxiety	-.029	-.309	-.033	-.221
Whole Fruits	-.124	-.179	-.153	.339
HEI	.131	.374	.118	-.123
Sleep Time	-	-	-.002	
R^2	.569		.579	
Adjusted R^2	.446		.432	
Significance	.004		.491	

Table 16. Hierarchical Linear Regression Model for the Entire Population using Simpson Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	.986		1.190	
Gender	.059	.183	.075	.230
Ethnicity	-.086	-.255	-.086	-.255
Batch Number	.086	.263	.076	.233
BMI	-.014	-.212	-.015	-.222
Anxiety	-.001	-.046	-.001	-.047
Whole Fruits	-.016	-.090	-.018	-.099
HEI	.027	.282	.024	.258
Sleep Time	-	-	.000	-.139
R^2	.292		.306	
Adjusted R^2	.187		.186	
Significance	.017		.339	

Table 17. Hierarchical Linear Regression Model for Female Participants using Simpson Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	1.065		1.826	
Ethnicity	-.181	-.526	-.190	-.550
Batch Number	.187	.553	.170	.502
BMI	-.013	-.167	-.019	-.247
Anxiety	.007	.363	.009	.464
Whole Fruits	.052	.306	.070	.411
HEI	-.015	-.148	-.034	-.326
Sleep Time	-	-	-.001	-.409
R^2	.418		.559	
Adjusted R^2	.243		.396	
Significance	.066		.024	

Table 18. Hierarchical Linear Regression Model for Male Participants using Simpson Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	1.391		1.659	
Ethnicity	-.086	-.265	-.093	-.288
Batch Number	.051	.163	.036	.117
BMI	-.022	-.333	-.022	-.327
Anxiety	-.012	-.478	-.013	-.522
Whole Fruits	-.023	-.127	-.033	-.178
HEI	.016	.169	.012	.127
Sleep Time	-	-	-.001	-.148
R^2	.609		.624	
Adjusted R^2	.497		.493	
Significance	.002		.381	

Table 19. Hierarchical Linear Regression Model for the Entire Population using Inverse Simpson Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	4.687		7.476	
Gender	3.787	.345	3.995	.364
Ethnicity	-.452	-.040	-.449	-.039
Batch Number	3.856	.350	3.721	.337
BMI	-.303	-.133	-.312	-.137
Anxiety	-.044	-.060	-.045	-.061
Whole Fruits	.337	.056	.313	.052
HEI	.798	.249	.767	.240
Sleep Time	-	-	-.006	-.056
R^2	.285		.287	
Adjusted R^2	.178		.163	
Significance	.020		.702	

Table 20. Hierarchical Linear Regression Model for Female Participants using Inverse Simpson Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	8.044		25.153	
Ethnicity	-1.763	-.144	-1.948	-.159
Batch Number	5.373	.446	4.987	.414
BMI	-.183	-.068	-.318	-.118
Anxiety	.094	.134	.139	.198
Whole Fruits	3.326	.553	3.727	.620
HEI	-.496	-.135	-.912	-.248
Sleep Time	-	-	-.027	-.259
R^2	.389		.445	
Adjusted R^2	.205		.240	
Significance	.096		.181	

Table 21. Hierarchical Linear Regression Model for Male Participants using Inverse Simpson Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	12.084		19.817	
Ethnicity	-2.163	-.226	-2.380	-.249
Batch Number	2.599	.284	2.183	.238
BMI	-.354	-.178	-.344	-.173
Anxiety	-.140	-.187	-.171	-.230
Whole Fruits	-2.064	-.381	-2.334	-.431
HEI	.936	.343	.824	.301
Sleep Time	-	-	-.015	-.144
R^2	.412		.427	
Adjusted R^2	.244		.226	
Significance	.059		.487	

Table 22. Hierarchical Linear Regression Model for the Entire Population using Number of OTUs as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	53.957		73.824	
Gender	9.358	.182	10.838	.211
Ethnicity	-4.662	-.087	-4.644	-.087
Batch Number	18.490	.359	17.531	.340
BMI	-.610	-.057	-.676	-.064
Anxiety	.203	.059	.200	.058
Whole Fruits	8.030	.287	7.862	.281
HEI	1.620	.108	1.399	.093
Sleep Time	-	-	-.040	-.085
R^2	.256		.261	
Adjusted R^2	.145		.132	
Significance	.042		.567	

Table 23. Hierarchical Linear Regression Model for Female Participants using Number of OTUs as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	123.626		202.199	
Ethnicity	.100	.002	-.753	-.013
Batch Number	24.579	.432	22.804	.401
BMI	-2.898	-.228	-3.518	-.277
Anxiety	.549	.165	.755	.227
Whole Fruits	10.016	.352	11.856	.417
HEI	-4.166	-.240	-6.073	-.350
Sleep Time	-	-	-.126	-.251
R^2	.286		.339	
Adjusted R^2	.072		.096	
Significance	.288		.231	

Table 24. Hierarchical Linear Regression Model for Male Participants using Number of OTUs as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	39.513		20.549	
Ethnicity	-12.110	-.255	-11.579	-.244
Batch Number	15.270	.336	16.291	.358
BMI	-.217	-.022	-.241	-.024
Anxiety	.393	.106	.470	.127
Whole Fruits	7.581	.282	8.243	.307
HEI	4.833	.356	5.109	.377
Sleep Time	-	-	.038	.071
R^2	.481		.485	
Adjusted R^2	.333		.305	
Significance	.020		.716	

Table 25. Hierarchical Linear Regression Model for the Entire Population using Fisher Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	8.821		12.760	
Gender	1.942	.179	2.235	.207
Ethnicity	-.906	-.081	-.902	-.080
Batch Number	3.865	.356	3.675	.338
BMI	-.127	-.057	-.140	-.063
Anxiety	.038	.052	.037	.051
Whole Fruits	1.841	.312	1.808	.307
HEI	.230	.073	.187	.059
Sleep Time	-	-	-.008	-.080
R^2	.254		.259	
Adjusted R^2	.143		.130	
Significance	.043		.590	

Table 26. Hierarchical Linear Regression Model for Female Participants using Fisher Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	23.527		39.797	
Ethnicity	-.003	.000	-.179	-.014
Batch Number	5.210	.429	4.842	.399
BMI	-.606	-.224	-.734	-.271
Anxiety	.116	.164	.159	.224
Whole Fruits	2.333	.384	2.714	.447
HEI	-1.028	-.277	-1.423	-.384
Sleep Time	-	-	-.026	-.244
R^2	.292		.342	
Adjusted R^2	.080		.100	
Significance	.272		.244	

Table 27. Hierarchical Linear Regression Model for Male Participants using Fisher Index as a Dependent Variable

Variables	Model 1		Model 2	
	B	β	B	β
(Constant)	5.895		2.080	
Ethnicity	-2.446	-.249	-2.339	-.238
Batch Number	3.178	.338	3.383	.360
BMI	-.048	-.024	-.053	-.026
Anxiety	.072	.095	.088	.115
Whole Fruits	1.715	.309	1.849	.333
HEI	.913	.326	.969	.345
Sleep Time	-	-	.008	.069
R^2	.475		.478	
Adjusted R^2	.324		.295	
Significance	.023		.725	

Anthropometric and Body Composition Variables and their Association to Sleep

Figure 6, 7 and 8 show gender-stratified participants distribution according to their fat mass variation and sleep time duration. In female participants a significant positive correlation between fat mass percentage and average sleep time (r= .380, p= .050) as well as sleep quality (r= .453, p= .018) were observed. A significant positive correlation was also found between fat mass and sleep quality (r= .388, p= .046).

In male participants, a significant positive correlation was found between fat mass percentage and average sleep time as well as weekdays sleep time (r= .420, p= .026, r= .444, p= .018, respectively). Likewise, a significant positive correlation was found between fat mass and average sleep time (r= .391, p= .040). Weekend sleep time showed a significant negative correlation with bone mineral density (r= .418, p= .027) only in male participants (Table 31).

Physical Activity and its Association to Sleep

Gender-stratified sleep indicators did not show any significant correlation with physical activity variables such as: energy expenditure, total time performing MVPA, MVPA including only bouts longer than 10 minutes, number of steps per day, sedentary time including sleep, and sedentary time other than sleep (Table 32).

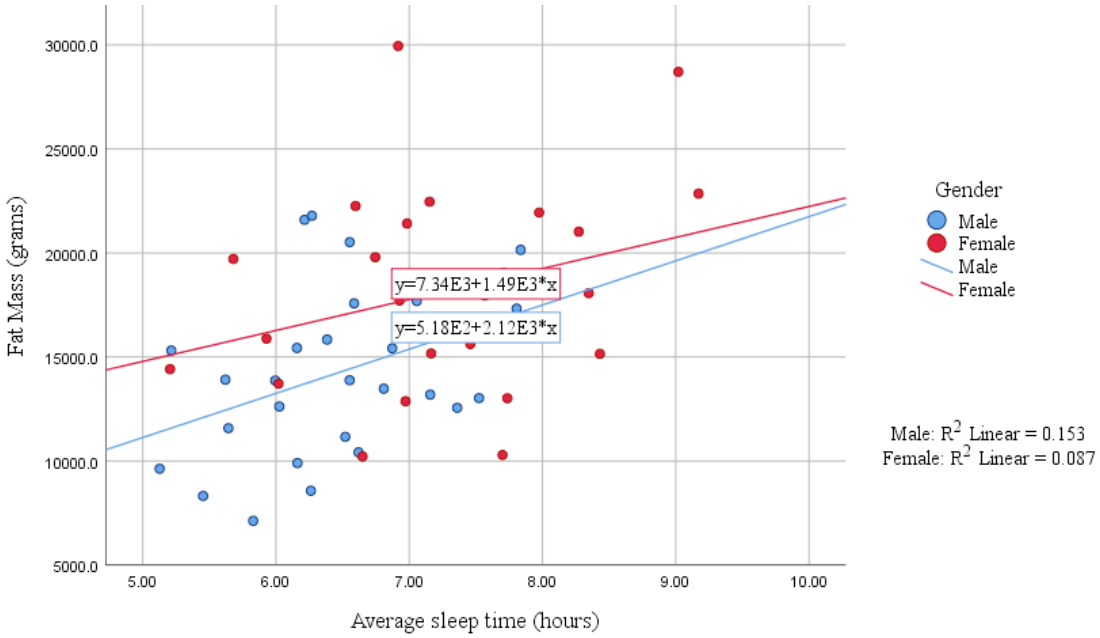

Figure 6. Scatterplot with Fit Line of Fat Mass by Sleep Time by Gender

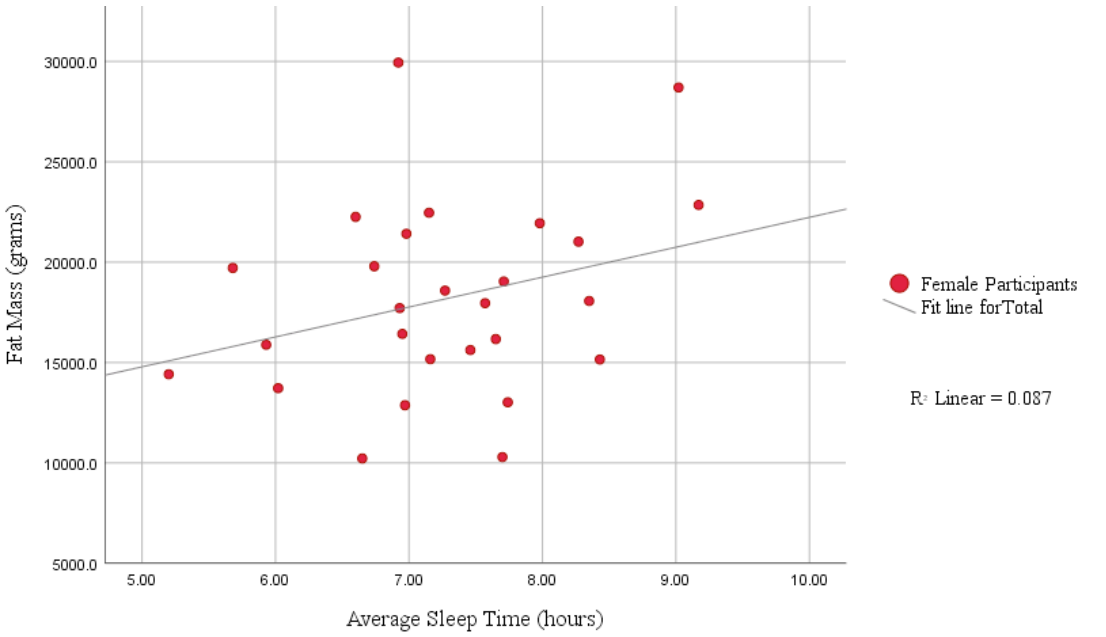

Figure 7. Scatterplot with Fit Line of Fat Mass by Sleep Time in Female Participants

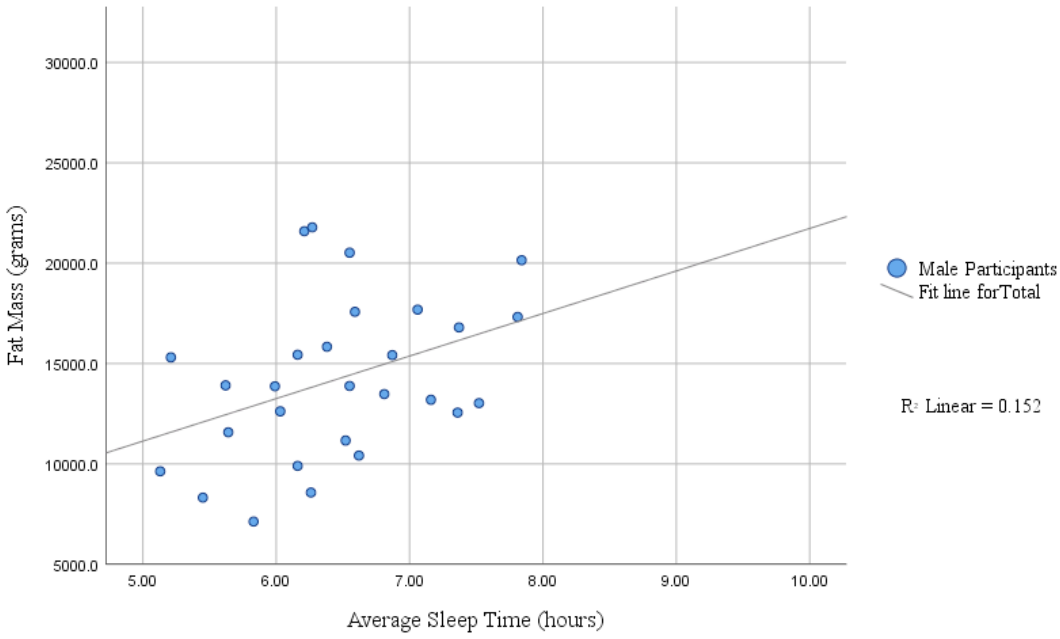

Figure 8. Scatterplot with Fit Line of Fat Mass by Sleep Time in Male Participants

Table 28. Correlation between Dietary and Sleep Variables

Variables	Parameters	Sleep time	SD of sleep	Weekdays sleep	Weekend sleep	Sleep Quality
		Female (n= 27)				
Calories Consumed	Correlation	.017	.104	.000	-.112	-.197
	Sig. (2-tailed)	.932	.606	.999	.579	.326
Corrected Carbohydrates[1]	Correlation	-.080	-.245	-.106	.013	-.218
	Sig. (2-tailed)	.693	.218	.598	.949	.276
Corrected Fiber[1]	Correlation	-.075	.051	-.004	-.156	-.412*
	Sig. (2-tailed)	.708	.799	.983	.438	.033
Corrected Protein[1]	Correlation	-.134	-.042	-.122	-.062	-.251
	Sig. (2-tailed)	.506	.837	.545	.760	.207
Corrected Fat[1]	Correlation	.216	.216	.303	-.055	.252
	Sig. (2-tailed)	.280	.279	.125	.784	.205
		Male (n= 28)				
Calories Consumed	Correlation	-.103	-.418*	-.012	-.133	.126
	Sig. (2-tailed)	.603	.027	.952	.501	.532
Corrected [illegible][1]	Correlation	-.114	.275	-.176	.040	.055
	Sig. (2-tailed)	.562	.157	.370	.840	.784
Corrected Fiber[1]	Correlation	-.069	.265	-.145	.142	-.076
	Sig. (2-tailed)	.729	.173	.461	.472	.708
Corrected Protein[1]	Correlation	.031	.145	-.134	.189	-.060
	Sig. (2-tailed)	.874	.463	.497	.335	.765
Corrected Fat[1]	Correlation	.014	-.297	.127	-.061	-.187
	Sig. (2-tailed)	.943	.124	.521	.759	.349

[1] Carbohydrates, Protein, Fat and Fiber consumption were corrected per every 1,000 of consumed calories
* Correlation is significant at the 0.05 level (2-tailed).

Table 29. Correlation between Healthy Eating Index and Sleep Variables in Female Participants

Variables	Parameters	Sleep time	SD of sleep	Weekdays sleep	Weekend sleep	Sleep Quality
Total Fruits	Pearson Correlation	-.113	.235	-.090	-.066	-.168
	Sig. (2-tailed)	.574	.238	.657	.743	.401
Whole Fruits	Pearson Correlation	-.016	.316	.058	-.109	-.198
	Sig. (2-tailed)	.937	.108	.774	.588	.323
Total Vegetables	Pearson Correlation	-.212	.118	-.263	.107	-.013
	Sig. (2-tailed)	.288	.556	.185	.594	.948
Green and Beans	Pearson Correlation	-.271	.038	-.135	-.424*	-.255
	Sig. (2-tailed)	.172	.851	.503	.028	.199
Whole Grains	Pearson Correlation	-.085	-.116	-.156	.173	-.322
	Sig. (2-tailed)	.674	.563	.436	.387	.101
Dairy	Pearson Correlation	.142	-.080	.141	.178	.197
	Sig. (2-tailed)	.481	.693	.482	.376	.324
Total Protein Food	Pearson Correlation	-.039	.286	-.045	-.065	-.417*
	Sig. (2-tailed)	.845	.148	.823	.748	.031
Seafood and Plant Proteins	Pearson Correlation	.052	-.295	.116	-.096	-.123
	Sig. (2-tailed)	.798	.136	.564	.633	.540
Refined Grains	Pearson Correlation	.174	.116	.174	-.086	.037
	Sig. (2-tailed)	.384	.565	.385	.671	.853
HEI Score	Pearson Correlation	-.213	-.001	-.168	-.122	-.234
	Sig. (2-tailed)	.285	.996	.403	.543	.239

Table 30. Correlation between Healthy Eating Index and Sleep Variables in Male Participants

Variables	Parameters	Sleep time	SD of sleep	Weekdays sleep	Weekend sleep	Sleep Quality
Total Fruits	Pearson Correlation	-.364	.028	-.260	-.346	.366
	Sig. (2-tailed)	.057	.888	.182	.072	.060
Whole Fruits	Pearson Correlation	-.355	.035	-.295	-.304	.303
	Sig. (2-tailed)	.064	.861	.127	.116	.125
Total Vegetables	Pearson Correlation	.299	.008	.183	.327	-.082
	Sig. (2-tailed)	.123	.970	.351	.090	.683
Green and Beans	Pearson Correlation	.137	.214	-.054	.391*	-.086
	Sig. (2-tailed)	.488	.274	.783	.040	.668
Whole Grains	Pearson Correlation	.012	-.300	.115	-.240	-.102
	Sig. (2-tailed)	.950	.121	.561	.218	.614
Dairy	Pearson Correlation	-.069	.252	-.099	-.128	.276
	Sig. (2-tailed)	.727	.195	.617	.516	.163
Total Protein Food	Pearson Correlation	.301	-.266	.229	.214	-.069
	Sig. (2-tailed)	.120	.171	.241	.275	.731
Seafood and Plant Proteins	Pearson Correlation	-.383*	-.197	-.279	-.274	-.057
	Sig. (2-tailed)	.044	.316	.151	.159	.778
Refined Grains	Pearson Correlation	-.119	-.116	-.084	-.146	.079
	Sig. (2-tailed)	.546	.555	.670	.458	.694
HEI Score	Pearson Correlation	-.140	.074	-.192	.009	-.032
	Sig. (2-tailed)	.479	.709	.327	.966	.873

* Correlation is significant at the 0.05 level (2-tailed).

Table 31. Correlation between Body Composition and Sleep Variables

Variables	Parameters	Sleep time	SD sleep	Weekdays sleep	Weekend sleep	Sleep Quality
		Female (n= 27)				
Fat Mass (gr)	Correlation	.295	-.159	.267	.211	.388*
	Sig. (2-tailed)	.136	.429	.178	.291	.046
Lean Mass (gr)	Correlation	-.254	-.225	-.238	-.217	-.282
	Sig. (2-tailed)	.201	.260	.232	.276	.154
Percentage Fat Mass (%)	Correlation	.380*	-.057	.350	.284	.453*
	Sig. (2-tailed)	.050	.779	.073	.151	.018
VAT area (cm2)	Correlation	.252	.071	.294	.034	.335
	Sig. (2-tailed)	.205	.726	.136	.867	.087
BMD (g/cm2)	Correlation	-.246	-.052	-.208	-.308	-.101
	Sig. (2-tailed)	.217	.797	.298	.118	.616
		Male (n= 28)				
Fat Mass (gr)	Correlation	.391*	-.122	.362	.229	-.006
	Sig. (2-tailed)	.040	.537	.058	.241	.976
Lean Mass (gr)	Correlation	-.116	.339	-.232	.051	.165
	Sig. (2-tailed)	.558	.077	.235	.797	.411
Percentage Fat Mass (%)	Correlation	.420*	-.259	.444*	.201	-.054
	Sig. (2-tailed)	.026	.183	.018	.304	.788
VAT area (cm2)	Correlation	.269	.105	.167	.362	.304
	Sig. (2-tailed)	.166	.597	.396	.058	.124
BMD (g/cm2)	Correlation	.186	.111	-.009	.418*	.021
	Sig. (2-tailed)	.343	.575	.962	.027	.918

* Correlation is significant at the 0.05 level (2-tailed).

Metabolic Syndrome and Cardiovascular Risk Factors and their Association to Sleep

Gender-stratified correlations between sleep variables and metabolic syndrome risk factors did not show any significant finding in female participants, however, five significant correlations were found in male participants. Specifically, significant positive correlations were found between weekly sleep time as well as weekdays sleep time with triglycerides (r= .546, p= .003; r= .576, p= .001, respectively). Sleep standard deviation showed a significant negative correlation with SBP (r= -.419, p= .027). Weekly sleep time and weekend sleep time showed significant negative correlations with DBP (r= -.389, p= .041; r= -.425, p= .024). HDL cholesterol, glucose, and waist c. did not show significant correlations with sleep variables (Table 33).

Anxiety and Depression and their Association to Sleep

Significant positive correlations between both depression and anxiety with sleep quality were observed in female participants (r= .450, p= .019, r= .480, p= .011, respectively). In male participants, a significant negative correlation was observed between sleep time and depression (r= -.441, p= .019) as well as anxiety (r= -.376, p= .049) (Table 34).

Comparison of Extreme Sleep Groups by Gender

Participants were first stratified by gender, then, ordered according to their sleep time, and divided into tertiles (McNeil, Doucet et al. 2013; Aili et al. 2015; Owens et al. 2016).

Table 32. Correlation between Physical Activity and Sleep Variables

Variables	Parameters	Sleep time	SD of sleep	Weekdays sleep	Weekend sleep	Sleep Quality
			Female (n= 27)			
Energy Expenditure (Kcal)	Correlation	-.338	-.171	-.291	-.289	-.041
	Sig. (2-tailed)	.084	.393	.142	.144	.841
Total PA (hours)	Correlation	-.342	.038	-.299	-.308	-.193
	Sig. (2-tailed)	.081	.852	.129	.118	.334
PA (including only bouts >10min)	Correlation	-.362	.022	-.299	-.358	-.287
	Sig. (2-tailed)	.064	.913	.130	.066	.147
Number of Steps	Correlation	-.334	-.140	-.254	-.357	.056
	Sig. (2-tailed)	.089	.486	.201	.068	.780
Sedentary Time (including sleep)	Correlation	.312	-.118	.240	.312	.166
	Sig. (2-tailed)	.121	.564	.237	.121	.419
Sedentary Time (not including sleep)	Correlation	-.210	-.271	-.261	.004	-.140
	Sig. (2-tailed)	.304	.181	.198	.983	.495
Male (n= 28)						
Energy Expenditure (Kcal)	Correlation	-.179	.176	-.275	.003	.062
	Sig. (2-tailed)	.363	.370	.157	.989	.759
Total PA (hours)	Correlation	-.172	.178	-.238	-.021	.029
	Sig. (2-tailed)	.381	.364	.222	.916	.886
PA (including only bouts >10min)	Correlation	-.145	.151	-.221	.006	.043
	Sig. (2-tailed)	.462	.443	.258	.976	.830
Number of Steps	Correlation	-.171	-.117	-.220	-.007	-.216
	Sig. (2-tailed)	.383	.555	.261	.973	.278
Sedentary Time (including sleep)	Correlation	.215	.016	.261	-.006	.202
	Sig. (2-tailed)	.292	.940	.198	.977	.334
Sedentary Time (not including sleep)	Correlation	-.107	.011	-.037	-.200	.311
	Sig. (2-tailed)	.603	.958	.858	.328	.131

Table 33. Correlation between Metabolic Syndrome and Sleep Variables

Variables	Parameters	Sleep time	SD of sleep time	Weekdays sleep time	Weekend sleep time	Sleep Quality
			Female (n= 27)			
TAG	Correlation	.205	-.275	.167	.199	.347
	Sig. (2-tailed)	.304	.166	.406	.320	.077
HDL	Correlation	.200	.023	.075	.377	.055
	Sig. (2-tailed)	.318	.911	.712	.053	.787
Glucose	Correlation	-.163	-.281	-.120	-.255	.206
	Sig. (2-tailed)	.418	.155	.552	.199	.302
SBP	Correlation	-.161	-.294	-.140	-.166	-.002
	Sig. (2-tailed)	.423	.137	.487	.408	.992
DBP	Correlation	.102	-.379	.110	.039	.026
	Sig. (2-tailed)	.613	.051	.585	.845	.899
Waist C. (cm)	Correlation	.020	-.199	.025	-.043	.120
	Sig. (2-tailed)	.922	.320	.903	.833	.553
			Male (n= 28)			
TAG	Correlation	.546**	-.275	.576**	.132	-.099
	Sig. (2-tailed)	.003	.156	.001	.504	.624
HDL	Correlation	-.058	.354	-.172	.214	.119
	Sig. (2-tailed)	.768	.064	.382	.274	.553
Glucose	Correlation	.144	-.149	.192	-.098	-.268
	Sig. (2-tailed)	.464	.450	.328	.618	.177
SBP	Correlation	-.292	-.419*	-.152	-.321	.027
	Sig. (2-tailed)	.131	.027	.441	.096	.895
DBP	Correlation	-.389*	.001	-.258	-.425*	.207
	Sig. (2-tailed)	.041	.996	.184	.024	.300
Waist C. (cm)	Correlation	.163	.159	.138	.050	.225
	Sig. (2-tailed)	.408	.420	.485	.799	.258

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table 34. Correlation between Psychological and Sleep Variables

Variables	Parameters	Sleep time	SD of sleep	Weekdays sleep	Weekend sleep	Sleep Quality
			Women (n= 27)			
Depression	Correlation	.343	.072	.366	.124	.450*
	Sig. (2-tailed)	.080	.722	.060	.536	.019
Anxiety	Correlation	.168	.026	.138	.111	.480*
	Sig. (2-tailed)	.401	.897	.491	.581	.011
			Men (n= 28)			
Depression	Correlation	-.441*	-.255	-.329	-.335	.254
	Sig. (2-tailed)	.019	.190	.088	.081	.201
Anxiety	Correlation	-.284	-.185	-.112	-.376*	.223
	Sig. (2-tailed)	.142	.347	.569	.049	.264

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Tables 35 and 36 show the comparison between the first and last tertiles of sleep time for female and male participants, respectively. These low and high sleep tertiles will be described from now on as extreme sleep groups. As expected, the comparison of extreme sleep groups in female participants showed a significant difference in weekly sleep time, and standard deviation of sleep ($p < .001$ and $p = .039$), being higher in long sleepers. Energy expenditure and number of steps in female participants were significantly lower in long-sleepers ($p = .039$, $p = .036$) in comparison to short sleepers. Long-sleepers female participants also showed significantly lower values for the alpha diversity indexes Shannon ($p = .032$) and Simpson ($p = .024$), as well as significantly lower fruits and vegetables consumption ($p = .044$). Correction for fruits and vegetables consumption erases, however, the relationship between sleep time and both alpha

diversity measures. The rest of the variables did not differ between the extreme sleep groups (Table 35). Consumption of fruit and vegetables were equally distributed among batches (p= 0.15).

Comparison of extreme sleep groups in male participants showed an expected significant difference in weekly sleep time (p <.001), being higher in long-sleepers. Long-sleepers had significantly higher fat mass (p= .018), fat mass percentage (p= .024), and fat mass to height ratio (p= .040) in comparison to their short-sleep counterparts. Triglycerides were significantly higher in long-sleepers versus short-sleepers (p= .037). Although depression tended to be higher in short-sleepers this relationship did not reach statistical significance (p= .062). The rest of the variables did not show any statistical differences between extreme sleep groups (Table 36).

Table 35. Comparison Between Extreme Sleep Groups in Female Participants

Variables	Short sleepers (n= 9)		Long sleepers (n= 9)		*p*
	Average	SD	Average	SD	
Psychological variables					
Anxiety (Beck Inventory)	10.78	11.60	8.89	7.01	.681
Depression (Beck Inventory)	6.22	7.05	8.00	6.08	.575
Body Composition					
Fat Mass (gr)	18183.71	5731.06	18897.00	5577.29	.792
Percentage Fat Mass (%)	28.09	6.81	30.36	5.72	.456
Fat Mass to Height ratio	6.63	1.95	6.86	1.76	.793
Lean Mass (gr)	43695.34	5904.27	40280.38	5516.25	.223
Diet					
Calories (kcal)	1778.81	684.45	1905.24	745.81	.713
Corrected Carbohydrates[1] (gr)	123.71	24.49	114.79	24.90	.455
Corrected Fiber[1] (gr)	11.09	8.13	7.86	2.84	.277
Corrected Protein[1] (gr)	48.50	17.72	44.57	16.24	.631
Corrected Fat[1] (gr)	37.96	10.70	39.44	10.93	.776
Fruits & Veg. [1] (portions/day)	3.14	2.43	1.22	0.98	.044*
HEI food groups	5.33	1.70	4.11	1.62	.149
Sleep					
Weekly Average Sleep (h)	6:18	0:34	8:16	0:33	.000*
SD of Sleep (h)	1:10	0:13	1:35	0:15	.039*
Physical Activity					
Average METs	1.4825	.18383	1.4100	.19242	.441
Sedentary and Light PA (0 to < 3 METs)	16.3478	6.50666	19.9833	2.63632	.140
Moderate PA (≥ 3 to < 6 METs)	1.6233	.82347	1.4300	.85051	.631
Vigorous PA (>6 METs)	.1456	.2140	.0611	.0611	.287
MVPA (> 3 METs)	1:59	0:40	1:31	0:58	.239
MVPA bout >10m	1:16	0:35	0:43	0:47	.122
Number of Steps	9476.67	2835.99	6712.89	2238.13	.036*
Energy Expenditure (Kcal)	2259.89	234.76	2018.67	219.65	.039*
Sedentary Time w/sleep (h:min)	17:43	1:34	18:24	1:47	.424
Sedentary Time no sleep (h:min)	11:15	1:24	10:17	1:40	.221
Blood Markers and BP					
Glucose (mg/dl)	92.56	3.84	91.00	4.47	.440
Triglycerides (mg/dl)	90.56	34.62	88.00	46.87	.897
Cholesterol (mg/dl)	172.89	40.41	165.22	26.65	.641
HDL (mg/dl)	57.78	11.11	66.67	18.21	.229
LDL (mg/dl)	96.89	29.99	81.00	20.74	.210
Systolic BP (mmHg)	106.56	9.46	100.22	9.42	.174
Diastolic BP (mmHg)	72.11	5.33	70.33	7.05	.555
Alpha Diversity					
Observed OTU	93.89	28.51	78.00	33.66	.296
Shannon	2.98	0.48	2.17	0.93	.032*
Simpson	0.90	0.05	0.69	0.24	.024*
Inv Simpson	11.86	6.17	6.60	6.27	.091
Fisher	16.81	6.18	13.58	7.28	.324

[1] Carbohydrates, Protein, Fat and Fiber consumption were corrected per every 1,000 of consumed calories.
* Correlation is significant at the 0.05 level (2-tailed).
*** Correlation is significant at the 0.001 level (2-tailed).

Table 36. Comparison Between Extreme Sleep Groups in Male Participants

Variables	Short sleepers (n= 9)		Long sleepers (n= 9)		*p*
	Average	SD	Average	SD	
Psychological variables					
Anxiety (Beck Inventory)	8.89	4.99	6.00	4.53	.216
Depression (Beck Inventory)	8.33	6.24	3.67	3.08	.062
Body Composition					
Fat Mass (gr)	11977.12	3028.32	15514.07	2635.50	.018*
Percentage Fat Mass (%)	15.96	3.86	20.32	3.57	.024*
Fat Mass to Height ratio	3.73	1.05	4.74	0.85	.040*
Lean Mass (gr)	60857.97	9905.24	58856.73	10571.55	.684
Diet					
Calories (kcal)	2823.88	800.82	2520.60	476.33	.343
Corrected Carbohydrates[1] (gr)	111.17	24.61	105.84	16.33	.596
Corrected Fiber[1] (gr)	9.96	3.75	8.27	2.04	.252
Corrected Protein[1] (gr)	52.62	14.37	50.36	21.31	.795
Corrected Fat[1] (gr)	42.59	11.24	43.00	9.22	.932
Fruits & Veg. [1] (portions/day)	2.33	2.06	2.22	2.06	.910
HEI Food Groups	6.33	1.255	5.56	1.67	.276
Sleep					
Weekly Average Sleep (h)	5:40	0:22	7:19	0:22	.000***
SD of Sleep (min)	1:02	0:11	1:08	0:09	.447
Physical Activity					
Average METs	1.7700	.26463	1.6556	.18242	.311
Sedentary and Light PA (0 to < 3 METs)	15.9789	6.20753	17.9211	3.13330	.414
Moderate PA (≥ 3 to < 6 METs)	2.4767	1.67178	2.5911	.94758	.860
Vigorous PA (>6 to <9 METs)	.3850	.4032	.1744	.1318	.158
MVPA (> 3 METs)	2:56	1:33	2:45	1:02	.767
MVPA bout >10m	2:11	1:26	2:00	1:02	.761
Number of Steps	9749.78	4365.32	9121.33	3449.39	.739
Energy Expenditure (Kcal)	3138.11	710.72	3014.33	423.47	.660
Sedentary Time w/sleep (h:min)	16:41	2:15	16:54	2:05	.844
Sedentary Time no sleep (h:min)	10:57	2:23	9:41	2:00	.235
Blood Markers and BP					
Glucose (mg/dl)	97.22	5.21	100.67	8.67	.322
Triglycerides (mg/dl)	67.89	19.19	87.78	17.88	.037*
Cholesterol (mg/dl)	154.56	24.38	157.78	9.92	.718
HDL (mg/dl)	52.22	11.64	50.44	9.91	.732
LDL (mg/dl)	88.67	20.97	89.67	12.20	.903
Systolic BP (mmHg)	115.33	5.72	110.78	9.96	.305
Diastolic BP (mmHg)	75.78	9.44	71.00	8.08	.266
Alpha Diversity					
Observed OTU	86.44	24.44	79.22	25.75	.550
Shannon	2.64	0.48	2.54	0.51	.657
Simpson	0.83	0.10	0.82	0.11	.751
Inv Simpson	8.24	5.43	7.83	4.93	.870
Fisher	15.18	5.13	13.71	5.36	.558

[1] Carbohydrates, Protein, Fat and Fiber consumption were corrected per every 1,000 of consumed calories.
* Correlation is significant at the 0.05 level (2-tailed).
*** Correlation is significant at the 0.001 level (2-tailed).

CHAPTER FIVE

Discussion

Main Hypothesis: There Will Be A Significant Positive Relationship Between Sleep Time And Quality With Alpha Diversity.

Recent research analyzing the effect of sleep duration on the human gut microbiota has found conflicting results. Comparison between either two days sleeping for only 4.25h/day versus sleeping for 8.5h/day revealed that after two days of sleep deprivation the gut microbiota showed a significant higher abundance of *Coriobacteriaceae* and *Erysipelotrichaceae*, with a lower concentration of *Tenericutes*, all of which have been previously associated with metabolic perturbations (Benedict, Vogel et al. 2016). Whereas, a similar study, but with a longer sleep deprivation period (4h/night for 5 days), failed to observe significant alterations in the human gut microbiota (Zhang, Bai et al. 2017).

Although we observed a significant negative correlation between weekdays sleep time and alpha diversity in female participants, this relationship was nullified after adjusting for fruit and vegetables consumption, which was higher in female short-sleepers. No significant relationships between sleep and alpha diversity parameters were found in male participants. Multivariable linear regression analysis did not show any sleep variables as strong predictors of alpha diversity. In addition, hierarchical linear models predicting alpha diversity showed sleep to be an important predictor only in models run in female population but not in males or the entire population. It is possible that these results are due to the difference in strength of the initial models, thus, adding an

extra variable might not have a significant impact in the stronger males' model but it could have an impact in the weaker females' model. This discrepancy by gender could be caused by the higher variability in parameters collected from female participants, creating weak models that were significantly improved by adding sleep.

Although analysis of microbial composition and function is needed before drawing further conclusions in regards to the effect of sleep on the gut microbiota, previous research has observed a lack of change in gut microbial composition in response to circadian disruption (Voigt, Forsyth et al. 2014). According to their research, the presence of a concomitant stress, such as consuming a poor diet (high fat, high sugar), is necessary to reveal the effect of circadian disruption on gut microbiota diversity and composition (Voigt, Forsyth et al. 2014). The lack of "challenging conditions" in our participants (healthy, young, normal BMI, with high physical activity levels, etc.) could work as a protective environment to revert some of the detrimental effects of sleep deprivation on alpha diversity and body composition previously observed in other populations.

Previous animal and human research has shown the protective effect that exercise has on sleep deprivation. In particular, human research has shown that acute exercise attenuates the increase in glucose, insulin, and free fatty acid produced by sleep deprivation (de Souza et al. 2017). Whereas animal research has shown a protective role of acute and chronic exercise on short and long-term memory, as well as anxiety, and depression caused by sleep deprivation (Vollert et al. 2011; Zagaar et al. 2012; Zagaar et al. 2013; Daniele et al. 2017).

Overall, our findings don't support our initial hypothesis as we did not observe strong, consistent relationships between sleep time and quality with alpha diversity measures; results that are in agreement with those demonstrated by Zhang et al. (Zhang, Bai et al. 2017). It is possible that the high physical activity levels of our population accompanied by the stringent selection criteria for healthy participants decreased the impact of sleep deprivation on health-related outcomes.

Gut Microbiota

Ethnicity has previously been shown to affect microbiota diversity and composition (Stearns et al. 2017). In our male population, being Caucasian increased Shannon index by .476 units; however, this relationship was not present in female participants. Although genetic factors can influence gut microbial composition (Spor et al. 2011), environmental factors seem to be the strongest features shaping the gut microbiome (Yatsunenko et al. 2012). A couple of possible explanations for the higher alpha diversity observed in Caucasian male participants in comparison to their male counterparts of other ethnicities could be having higher variety in their diets, being born vaginally, longer duration of breastfeeding, geographical location, etc. (Bokulich et al. 2016; Stearns, Zulyniak et al. 2017; Bowyer, Jackson et al. 2018). Although, we did not measure socioeconomic status, emerging research suggests that socioeconomic status has a stronger impact on gut microbiota than age, ethnicity or BMI (Miller, Engen et al. 2016). According to this research, the higher the socioeconomic status, the higher the alpha diversity, which can explain up to 22% of fluctuations in this index. It is possible that the driver of changes in the gut microbiota is not the socioeconomic status *per se* but the higher diversity in the diet, and/or higher physical activity levels in population with higher socioeconomic status

as these factors have been related to increased alpha diversity and changes in gut microbial composition (Clarke, Murphy et al. 2014; Bowyer, Jackson et al. 2018). Ethnicity is likely another mediating factor controlling the strong correlation between socioeconomic status and alpha diversity through both genetic and cultural effects (Spor, Koren et al. 2011; Yatsunenko, Rey et al. 2012).

Besides ethnicity, another factor that seems to play a pivotal role in gut microbiome structure is gender. Strong evidence supports the potential effect of sex-derived microbial differences explaining the discrepancy observed to develop autoimmune diseases, as 80% of patients affected by autoimmune diseases are women (Markle et al. 2014). Cumulative evidence demonstrates the striking sex differences in gut microbial composition that become particularly evident after the pubertal period (Markle et al. 2013). It is precisely at pubertal time when the majority of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematous, scleroderma, and multiple sclerosis among others, first appear (Markle, Frank et al. 2014). Animal research demonstrates that germ-free mice do not present the gender bias risk for autoimmune diseases, thus, suggesting a potential bacterial role controlling autoimmune risk (Yurkovetskiy et al. 2013). Furthermore, animal research has shown a decreased protection by male mice when gonadectomy is performed, as well as increased female protection to autoimmunity when hormonally treated with androgens (Yurkovetskiy, Burrows et al. 2013; Markle, Frank et al. 2014). Transplant of male microbiome into pre-pubertal females induces a sustained increase in testosterone that prevents the appearance of autoimmunity (Markle, Frank et al. 2014). Recent publications suggest a potential mechanism of action involving specific strains of gut bacteria reconverting bile-derived cholesterol metabolites into androgens, which

suggests a bi-directional communication between androgens and the gut microbiome (Gomez et al. 2015). Although, immunity is far from the scope of this study, the results derived from this research, clearly manifest a sex-specific gut microbiome. As previously described "Sex is an important factor to consider when looking at interactions between gut microbiome and environmental factors" (Org, Mehrabian et al. 2016). Due to this evidence, we decided to divide our total number of participants by sex to 27 female and 28 male participants, respectively.

H1: There Will Be A Significant Relationship Between Dietary Parameters And Alpha Diversity.

Previous research has shown caloric content and macronutrient distribution to account for the majority of alterations in the gut microbial composition (Graham, Mullen et al. 2015). Although we have only analyzed alpha diversity indexes and not microbial composition, we were not able to see correlations between caloric intake or macronutrient composition with alpha diversity measures. This lack of relationship could be caused due to the short time frame of dietary data collection (a single 24h food log). It is possible that although participants were instructed to avoid changing their diet through the duration of the study, collecting several days of food data would have provided a more accurate representation of their habitual diets. Collecting a Food Frequency Questionnaire could be another option to measure long-term habitual food intake, however, food frequencies do not provide an accurate representation of the eating behavior immediately before the stool sample collection, which may diverge from typical eating behavior. We recommend future studies to collect both habitual diet and acute diet immediately prior the stool sample collection.

Even though alpha diversity was not correlated to the caloric or macronutrient composition of the diet, it showed positive correlations to other dietary variables including total fruits, whole fruits, dairy, and refined grains, as well as a negative correlation with seafood. We observed a higher strength in the correlation between alpha diversity and whole fruits in comparison to the correlation between alpha diversity and total fruits. Our total fruits variable included juices, whereas the whole fruits variable included only intact fruits. Fruit juice has a high amount of naturally occurring sugar without the fiber content of the whole food, which could affect in a slightly differential way gut microbiome. Fiber molecules such as fructo-oligosaccharides, present in fruits, vegetables, and some grains, have shown to produce SCFA, which play an important role preventing bowel disease, colon cancer, and metabolic syndrome (Sawicki et al. 2017).

In regards to the observed positive association between dairy and alpha diversity, previous research has also found this association with both fermented and enriched dairy products (Sadrzadeh-Yeganeh et al. 2010; Wang et al. 2012; Kato-Kataoka et al. 2016). Our dairy products variable did not differentiate between fermented and non-fermented products, however, careful re-examination of the data demonstrated that the majority of the participants reporting consuming dairy products were in fact ingesting some type of fermented food. From our total of 55 participants, 40 reported to consume some type of dairy product. From these 40 participants, 22 expressed to consume at least 1 portion of cheese, 18 participants reported to consume at least 1 portion of yogurt, and 4 participants stated to consume at least 1 portion of sour cream. No participant reported eating other dairy fermented foods such as: kefir, probiotic yogurt, raw cheese, or miso.

The association between alpha diversity and seafood has not been previously reported. In contrast to the positive associations between alpha diversity and other food groups, we observed a negative association between alpha diversity and seafood. A recent crossover study showed a differential modulation of the gut microbiome by 4 weeks of lean-seafood versus non-seafood diets. Specifically, consuming lean seafood for a month increased up to two times the amount of circulating TMAO, which is a metabolite that has been linked to cardiovascular disease risk. TMAO concentration in blood was positively correlated to fecal TMAO and specific gut bacteria belonging to the order of *Clostridiales*, *Desfulfovibrionales*, and *Erysipelotrichales* (Schmedes et al. 2018). The specific long-term effects of high levels of TMAO in blood are still under debate.

In agreement with previous research (Guenther, Casavale et al. 2013), our population revealed a positive association between the number of food groups according to the HEI and alpha diversity. This relationship reflects the importance of keeping variety in the diet in order to increase microbial richness and abundance in the gut, which has been linked to positive health outcomes (Bermon et al. 2015). When using multivariable linear regressions to create models that explain alpha diversity, dietary variables were among the strongest predictors. Overall, the variables fruits and vegetables, seafood, and number of food groups (HEI) were all strong predictors of microbiota alpha diversity. In accordance to previous research, our results demonstrate that the HEI has a stronger effect on alpha diversity than BMI (Davis et al. 2017). Sex-stratified, extreme sleep groups comparison showed a significantly higher alpha diversity in female short-sleepers in comparison to their long-sleep counterparts. The higher alpha diversity in female short sleepers was likely caused by the higher intake of fruits and vegetables by this subgroup.

Correcting for fruits and vegetables consumption totally erased the correlation between sleep and alpha diversity.

Overall, we accepted our hypothesis as we observed several important relationships between dietary variables and alpha diversity parameters. These relationships strongly support the historic dietary recommendation of keeping a varied diet to improve health (HHS 1995; CDC 2016), which according to our data, might be mediated by positive changes in the gut microbiome diversity.

H2: There Will Be A Significant Relationship Between Body Composition And Metabolic Syndrome Parameters With Alpha Diversity.

Previous research has observed a negative correlation between BMI and alpha diversity even after controlling for the confounding variable of nutritional intake (Yun et al. 2017). This relationship is comparable to our results observed in male participants. When creating models to explain alpha diversity variability, BMI also resulted as a strong predictor when analyzing the entire population and male participants. These outcomes demonstrate that the higher the BMI the lower the alpha diversity, which seems to be detrimental for health-related outcomes (Bermon, Petriz et al. 2015).

Even though we observed a significant negative relationship between BMI and alpha diversity, body composition parameters, such as fat mass, did not show any significant correlation with alpha diversity measures. Gut microbial composition has shown conflicting results in regards to its relationship to fat mass (Turnbaugh et al. 2009; Finucane et al. 2014; Kasai et al. 2015). Either a positive (Kasai, Sugimoto et al. 2015), negative (Turnbaugh, Hamady et al. 2009), or a lack of relationship (Finucane, Sharpton et al. 2014) between fat mass and alpha diversity have been observed. However, not

observing significant correlations between fat mass and alpha diversity measures does not preclude our data from experiencing specific strain associations with fat mass parameters (Finucane, Sharpton et al. 2014).

Significant positive correlations between LDL cholesterol and alpha diversity measures were observed in our population. Previous research has shown a lower alpha diversity in hypercholesterolemic subjects, which seems to be in disagreement with our results (Rebolledo, Cuevas et al. 2017). However, it is important to highlight several differences among the studies. First, Rebolledo *et al* compared a hypercholesterolemic group with a control group, whereas in our study any participant could be categorized as hypercholesterolemic according to the ATPIII classification (Huang 2009). Additionally, we recruited only healthy participants that had a lower BMI and were ~40y younger in comparison to Rebolledo's population. It is also possible that microbial composition, rather than alpha diversity, is the factor regulating the association between alpha diversity and LDL cholesterol, as it has been demonstrated that acetate, one of the SCFA produced by intestinal bacteria has shown to possess hypercholesterolemic effect (He et al. 2017). This could mean that having high levels of acetate-producing bacteria could potentially increase LDL cholesterol. However, this premise should be taken with caution until further analysis of microbial composition is performed.

Previous research has demonstrated a link between gut microbiota dysbiosis and hypertension in both animals and humans (Mell et al. 2015; Yang et al. 2015). In our study, we did not see significant correlations between alpha diversity and SBP; however, we observed a significant positive correlation between DBP and alpha diversity in our male participants. Although previous research has shown a lower alpha diversity in

hypertensive animal and humans (Yang et al. 2015), it is possible that it is not the bacterial diversity but the actual bacterial composition that is the important factor regulating the relationship between alpha diversity and blood pressure. Animal models of hypertension have shown to have significantly higher Firmicutes to Bacteroidetes ratio, higher butyrate and acetate but lower lactate-producing bacteria and depleted *Bifidobacteria* (Yang et al. 2015). Factors that are corrected after 4 weeks of antibiotic treatment, which significantly decreased blood pressure (Yang et al. 2015). Similar results showing decreasing blood pressure were found in a treatment-resistant patient when treated with a combination of antibiotics (Qi, Aranda et al. 2015). One of the proposed mechanism of action mediating the relationship between the gut microbiome and hypertension are the possible role of SCFA regulating receptors that control blood pressure. Animal research has shown SCFA to activate both the Olfr78 receptor in the juxtaglomerular apparatus where it induces renin production, and the Gpr41 receptor in small vessels, to produce vasodilation (Pluznick et al 2013). The net effect of activation of these two opposing mechanisms is a net null effect, however, it is possible that a disarrangement in one of these mechanisms leads to alterations in blood pressure. In addition, it is possible that the absence of beneficial bacterial could exacerbate the increase in blood pressure. Human research has shown that probiotic treatment (*Lactobacillus helveticus* and *Saccharomyces cerevisiae*) is able to reduce SBP and DBP in hypertensive patients (Hata, Yamamoto et al. 1996; Khalesi, Sun et al. 2014). A proposed mechanism of action through which probiotics decrease blood pressure is by the production of ACE-inhibitor molecules through the proteolysis and fermentation of fermented milk proteins (Gonzalez-Gonzalez, Gibson et al. 2013).

Altogether, we accept our hypothesis as we observed a significant correlation between alpha diversity and metabolic syndrome indicators but not with body composition parameters. Specifically, BMI seems to negatively correlate with alpha diversity, and positively with LDL cholesterol and DBP. These last two relationships could be explained by changes in microbial composition rather than changes in gut diversity.

H3: There Will Be A Significant Positive Relationship Between Physical Activity And Alpha Diversity.

Previous research has shown a significantly higher gut microbiota diversity in active population, which is accompanied by changes in microbial structure (Clarke, Murphy et al. 2014; Bressa et al. 2017). A significant negative association between sedentary time and alpha diversity has also been demonstrated (Bressa et al. 2017). The high physical activity levels in this population could be accompanied by differential caloric and/or protein intake, which has shown to contribute to the higher alpha diversity (Clarke, Murphy et al. 2014).

In our study, no significant correlations were found between alpha diversity and physical activity indicators. Likewise, sedentary-related variables did not seem to be correlated to alpha diversity measures. It is important to highlight, however, that 95% of our population falls within the category of "highly active" as they performed $\geq$300 min of MVPA per week, with the remaining 5% performing between 150-300 min (Adabonyan et al. 2010). The high levels of physical activity with low inter-individual variation could have obscured the appearance of significant correlations with alpha diversity measures. An 8-week human study comparing three experimental groups, 1)

combined aerobic and resistance exercise training (3 times per week), 2) daily protein supplementation (30g of whey protein), and 3) combined exercise plus protein supplementation failed to observe a significant increase in alpha diversity in response to exercise (Cronin, Barton et al. 2018). Even though Cronin's study did not show a significant difference between groups they observed a non-significant trend of a higher alpha diversity in the gut microbiota of both of the groups that performed exercise. It is possible that a more intense exercise protocol or a higher training frequency or duration could have made this finding significant. Contrary to our study, the participants involved in Cronin's study were sedentary and overweight-obese, which could have provided more room for improvement in their gut microbiome in response to exercise, in comparison to our healthy population.

We reject our hypothesis as we did not see any significant correlation between physical activity and alpha diversity parameters. Comparing the results of our highly active participants with a non-active population could provide us a more accurate representation of the effect of physical activity on alpha diversity measures.

H4: There Will Be Significant Relationship Between Anxiety And Depression With Alpha Diversity.

Previous research has revealed associations between gut microbiota composition and probiotic consumption with psychological variables such as anxiety and depression (Slyepchenko, Carvalho et al. 2014; De Palma, Lynch et al. 2017; Wallace and Milev 2017). In fact, recent research suggests a communication between the intestine and the central nervous system by acting through the vagus nerve, link known as the gut-brain axis. According to this research, microbial-derived neurotransmitters are able to influence

brain functions (Kali 2016). The effect that environmental stressors have on the gut microbiome and thus, in neurological disorders seems to be sex-dependent (Bruce-Keller et al. 2017).

In agreement with these results, the present study showed a strong negative correlation between anxiety and three alpha diversity indexes in male participants (Miller, Engen et al. 2016). According to multivariable linear regression, anxiety remained as one of the strongest predictors of alpha diversity in our male population (Stearns, Zulyniak et al. 2017). This means that the higher the anxiety scores, the lower the gut microbial alpha diversity, which highlights the detrimental independent effect that anxiety can have in our gut microbiome.

We accept our hypothesis as we observed a significant correlation between alpha diversity parameters and anxiety but not depression.

Sleep

According to the NSF, the recommended sleep time for young adults (18-25y) is 7 to 9h per night, however, the amount of time people sleep is decreasing across the years (Foundation 2005a; Foundation 2015). Around 40% of adults in the United States sleep <7h, with 31% sleeping between 7-8h, and 26% sleeping >8h. In our study, 60% of the participants were considered as short sleepers (<7h), 30% as average sleepers (7-8h), and only 10% as long sleepers (>8h). The potential difference between our results and those from the NSF could be caused due to inclusion of a different age range. The NSF used an age range of >18y with an average age of 49.0y, while our study managed an age range of 18-25y with an average of 20.8 ± 1.7y. Another potential explanation for the more exacerbated results that we observed could be caused by the year at which the study was

conducted. It is possible that the tendency of decreasing sleep time through the years continued taking place reaching lower numbers in the actuality in comparison to 2005 (when the NSF conducted their survey). The NSF has continued to perform new evaluations; however, the age range of the newer surveys does not include the age range of the present study and thus could not be used for comparison purposes.

Previous research has shown that more than 60% of students within the age of 17 to 24 years showed poor sleep quality according to the PSQI inventory (Lund, Reider et al. 2010). Our study results corroborate this finding having ~54.5% of our college students classified as having poor sleep quality.

H5: There Will Be A Significant Relationship Between Dietary Parameters And Sleep Time And Quality.

Previous research has shown increased energy intake, especially coming from carbohydrates, in response to sleep deprivation (Schiavo-Cardozo, Lima et al. 2013; Poggiogalle, Lubrano et al. 2016). In the present study we did not observe a significant correlation between sleep time and caloric or carbohydrate intake, however, we did observe a significant negative correlation between sleep standard deviation and caloric intake in male participants. According to our results the higher the day-to-day variation in sleep time the lower the caloric intake in male participants. In addition, a significant negative correlation between sleep quality and fiber intake took place in female participants, meaning that the higher the fiber intake, the lower the PSQI score which indicates better sleep quality. Extreme sleep group comparisons revealed a significant difference in fruits and vegetables consumption in female participants, indicating a higher intake in female short-sleepers versus their long-sleep counterparts. This significant

difference in fruits and vegetables consumption between extreme groups might be the explanation of the negative correlation between sleep quality and fiber intake observed in female participants.

The food group “greens and beans” presented a negative association with weekend sleep time in female participants but a positive association with weekend sleep time in male participants. This discrepancy is unclear as it was not present when comparing the average 7-day sleep time or average weekdays sleep time.

Interestingly, we observed a significant negative association between sleep quality and the food group “total protein” in female participants meaning that poorer sleep quality was related with lower protein consumption. A significant negative association between sleep time and the food group “seafood and plant proteins” (seafood, nuts, and seeds as well as soy products) was found in male participants. If we recall, a significant negative relationship was also found between “seafood and plant proteins” and alpha diversity. It could be possible that there is a relationship between the three variables (sleep, gut microbiota and the food group “seafood and plant proteins”) although, to our knowledge, no research to this date has shown such relationship.

We accept our hypothesis as we observed a significant relationship between standard deviation of sleep and caloric intake. We also observed significant correlations between sleep variables and the food groups: “greens and beans”, and “seafood and plant proteins”.

H6: There Will Be A Significant Relationship Between Body Composition And Metabolic Syndrome With Sleep Time And Quality.

Short and long sleepers have shown to have 58% and 47% higher waist circumference as well as 124% and a 94% higher body fat percentage in comparison to average sleepers (Chaput, Despres et al. 2008; Xiao, Arem et al. 2013). Although we were not able to observe a significant correlation between sleep time and waist circumference or visceral adipose tissue we did observe a significant positive correlation between sleep time and both body fat mass and percentage of fat mass. Therefore, the longer the sleep duration, the higher the fat mass of both female and male participants. However, we did not detect the U shape effect of sleep time on body fat mass that has been previously reported (Chaput, Despres et al. 2008; Xiao, Arem et al. 2013).

Although we did not measure disinhibition behavior in our study, it is possible that the lack of a higher body fat mass observed in our short sleep population could be explained by a lower disinhibition eating behavior. A disinhibited eating behavior represents a tendency towards overeating (mainly high-sugar, high-fat food) in response to cognitive or emotional cues. Research suggests that short sleepers who keep a low disinhibition eating behavior are not at higher risk to increase body fat than those participants with average sleep (Chaput, Despres et al. 2011a). Thus, if our short sleep population was formed by participants who are self-aware and have high self-control in regards to their eating behavior, it is likely that the short sleep itself wouldn't be able to produce an increase in fat mass. In addition, it is critical to keep in mind that only 11% of the participants in our population presented increased fat mass to height ratio.

In women, a significant positive correlation was found between fat mass percentage and both sleep time and sleep quality, meaning that longer sleep duration or poorer sleep

quality, were related to higher fat mass percentages, correlations that disappeared after correcting for MVPA time and lean mass (which tended to be higher in short sleepers). Likewise, in male participants, sleep time was also positively correlated to fat mass, which also disappeared after controlling for MVPA time and lean mass.

Lean mass has previously been shown to be negatively related to sleep time, which has been suggested to be caused by the lower physical activity levels and higher sedentary time (Kim et al. 2017b). Although no significant differences in the lean mass of our extreme groups were observed in the present study, it is important to highlight that female long-sleepers had 3.4 kg lower lean mass in comparison to their short-sleep counterparts, which could be explained by the higher MVPA levels (measured though energy expenditure and number of steps) observed in female short sleepers. Similarly, male long-sleepers showed on average 2.0 kg less lean mass in comparison to their short-sleepers counterpart.

Sleep time was positively correlated with BMD in male participants, which is in agreement with previous research revealing a decreased mineral bone density in short sleepers (Casazza et al. 2011; Fu et al. 2011; Cunningham et al. 2015). A potential explanation to the observed results may be the higher cortisol levels present in short-sleepers (Omisade, Buxton et al. 2010; Abell et al. 2016). Glucocorticoids have been shown to inhibit osteogenesis by reducing human bone marrow stromal cells proliferation (Carcamo-Orive et al. 2010) and by modifying the osteoclastic resorption mode from intermittent to continuous (Soe et al. 2010).

Body composition is not the only factor that has shown to be affected by sleep time. Short sleep duration has also been correlated with metabolic syndrome (Lin, Sun et al.

2016). Longitudinal studies have found a higher incidence of metabolic syndrome in individuals whose sleep duration decreased by ≥2h per night through a period of four years (Song, Liu et al. 2016). Although only one participant satisfied diagnosis for metabolic syndrome in our study, we observed several relationships between individual metabolic syndrome variables and sleep time. A significant negative correlation between sleep time and both SBP and DBP were observed. Previous research has suggested a mediator effect of the gut microbiome controlling the association between sleep deprivation and SBP (Durgan, Ganesh et al. 2016). Taking this research into consideration, we decided to control for alpha diversity when analyzing the correlation between sleep and SBP as well as DBP. Controlling for alpha diversity decreased the strength and significance of the correlation with SBP ($p = .049$) and totally erased the correlation with DBP.

An unexpected significant positive correlation between sleep time and TAG was found, which contrasts with what has been previously reported in older adults (aprox. 36 years of age)(Schiavo-Cardozo, Lima et al. 2013). This correlation decreased but remained significant after controlling for body fat mass (which was higher in the long-sleep population) and MVPA levels (which tended to be higher in the short-sleep population).

Overall, we accept our hypothesis as we observed several significant relationships between body composition and metabolic syndrome parameters with alpha diversity. Independently of gender, we observed a significant positive correlation between sleep time and fat mass, which seemed to be mediated by the lower MVPA time and lean mass of the population sleeping longer time. In addition, our data indicate, that the lower the

sleep duration, the higher the blood pressure, which may partially be modulated by alpha diversity in the gut. It is imperative to consider that both, the increase in fat mass and the increase in blood pressure as sleep time increases or decreases, respectively, were present within the acceptable range of both parameters. This is, the observed variation in fat mass as a response to sleep was present within the normal BMI range as we particularly excluded participants with a BMI> 27kg/m^2. Likewise, the variation in blood pressure occurred even though none of the participants satisfied criteria to be diagnosed as hypertensive.

H7: There Will Be A Significant Positive Relationship Between Physical Activity And Sleep Time And Quality.

Our male population performed ~1:30h more MVPA time per day in comparison to our female population, results that are in agreement to previous studies (Riso et al. 2018). Previous research has shown an average MVPA time of 38 minutes per day in an adult male population, whereas our younger college-aged male population performed 3:15h of daily MVPA (McClain et al. 2014). According to the CDC only 31% of adults meet 300 min of at least moderate physical activity per week (CDC 2014), whereas 32.8% female and 67.2% male college students adhere to these guidelines in the United States (Judge et al. 2012). In our college-age population, 83% of the participants met these guidelines. Physical activity was not considered as inclusion/exclusion criteria for the present study; however, we observed a significantly higher level of physical activity in our population in comparison to the national average or similar populations. Is likely that this higher percentage of physical activity is due to self-selection of the participants interested in our study and/or due to Baylor University requirements of taking several lifetime fitness

classes throughout the students' careers, the necessary walking commute through the large campus, as well as the high accessibility to courts, gyms, pools, and tracks at no added cost.

Previous human research has shown that reduction in sleep time decreases physical activity and energy expenditure (Nedeltcheva, Kilkus et al. 2010; McClain, Lewin et al. 2014; Hart, Hawley et al. 2017). Contrarily, non-sleep sedentary time has shown little to no association with physical activity (McClain, Lewin et al. 2014). In our study, we did not see any significant correlation between sleep measures and physical activity variables including sedentary behavior. Comparison between extreme sleep groups showed a significantly lower energy expenditure and number of steps in long-sleepers in female but not male participants. Previous research has also observed lower MVPA levels in adults sleeping more than 8h; however, this association was not present in younger adults (McClain, Lewin et al. 2014).

We do not accept our hypothesis as sleep time did not seem to strongly and consistently associate with the physical activity levels of our specific population. However, it is crucial to take into consideration the 95% of our population was considered as highly active which could have decreased the variation in this variable and prevented us from observing a significant correlation with sleep parameters. It is also possible that the cross-sectional nature of the study did not allow us to measure their physical activity in a more representative way, however, physical activity levels were objectively measured through 7 days by using a research-grade sleep monitor. Furthermore, participants were instructed to keep constant their regular physical activity patterns during the duration of the study.

H8: There Will Be A Significant Relationship Between Anxiety And Depression With Sleep Time And Quality.

Previous research has shown that sleep deprivation increases depression and anxiety risk (Nyer, Farabaugh et al. 2013; Jackson, Sztendur et al. 2014). Although conflicting research exists, studies have shown the predecessor effect of sleep abnormalities on depression and anxiety (Jackson, Sztendur et al. 2014). It is likely, however, that the relationship between sleep and psychological variables is bidirectional (Bruce-Keller, Fernandez-Kim et al. 2017).

In the present study we found a significant negative correlation between sleep time and depression in male participants which means the lower the sleep duration, the higher the levels of depression in male participants which is consistent to previous research (Zhai, Zhang, and Zhang, 2015). In female participants a positive correlation between sleep quality and depression was observed. If we keep in mind that higher PSQI levels mean poorer sleep quality, this correlation would mean that the lower the sleep quality, the higher the depression levels in female participants.

In addition, in male participants a significant negative relationship was observed between weekend sleep time and anxiety levels, meaning that the shorter the sleep duration on weekends the higher the anxiety levels. In female participants, a significant positive correlation was found between anxiety and sleep quality, which means that poor sleep quality was related to higher anxiety levels, as higher PSQI values indicate poorer sleep quality.

We accept our hypothesis as we observed significant correlations between anxiety and depression and sleep indicators. Particularly, we observed that poor sleep quality was

related to higher depression and anxiety in female participants, whereas short sleep duration was related to higher depression and anxiety levels in male participants.

Closing Remarks

Previous human research has found no significant effect of sleep deprivation on alpha diversity (Benedict, Vogel et al. 2016; Zhang, Bai et al. 2017). It is possible however, that the acute duration of these sleep interventions, along the low number of participants (one fifth of the total population used in the present study) could have decreased their chances of observing a significant difference between groups. According to a recent article, the estimated number of participants needed to detect between group differences of 15% in alpha diversity are 16 participants per group; to detect a difference of 10%, 35 participants; for 5% differences, 140 participants, and for 1%, 3,400 participants (Sze and Schloss 2016). In addition, according to our power analysis, a sample of at least 26 participants per group is needed in order to detect significant correlations with an effect size ≥ 0.5. Previous human research studies have used a sample size of 9 to 11 participants, whereas our study recruited a total of 55 participants, 27 female and 28 male participants (Benedict, Vogel et al. 2016; Zhang, Bai et al. 2017). The main goal of our study was to test the relationship between sleep duration and alpha diversity using a number of participants that would provide us enough power to detect significant correlations/differences. Furthermore, we sought to analyze the long-term effect of sleep duration on alpha diversity in a natural setting. In our population, under the circumstances of our participants, we did not observe a significant relationship between sleep duration and the gut microbiome. These results are consistent with what has been previously found in acute sleep deprivation studies (Benedict, Vogel et al. 2016;

Zhang, Bai et al. 2017). It is possible that the lack of relationship between sleep patterns and alpha diversity observed in our study was caused due to the lack of a secondary stressor as we recruited only healthy, young, normal BMI participants that were highly physically active (Voigt, Summa et al. 2016; Durgan 2017). It is also possible that the high physical activity levels of our population could have helped to alleviate the detrimental effects of short sleep on the gut microbiome as it has been previously demonstrated (Vollert et al. 2011; Zagaar et al. 2012; Zagaar et al. 2013; Daniele et al. 2017). We strongly recommend future researchers to focus on analyzing the effect of sleep on the gut microbiome in other less healthy/less athletic populations to evaluate if these results are preserved.

Some of the highlights observed in female participants are: 1) the positive effect of diet variety on gut microbial diversity, 2) the negative association between sleep quality and protein intake, 3) the stronger effect that diet has on alpha diversity in comparison to body composition, sleep time, or physical activity levels, 4) the potential mediator effect of physical activity and lean mass controlling the relationship between sleep time and fat mass, and 5) the direct relationship between poor sleep quality with depression and anxiety levels. On the other hand, the key remarks observed in male participants are: 1) the positive association between diet variety and gut microbial diversity, 2) the lower alpha diversity with higher BMI, 3) the lower alpha diversity with higher anxiety levels, 4) the negative association between depression and sleep duration, 5) the potential mediator effect of physical activity and lean mass controlling the relationship between sleep time and fat mass, and lastly 6) the negative correlation between short sleep and

blood pressure, as well as the potential mediator effect of gut diversity controlling this relationship.

The limitations of this study should be recognized when interpreting these results. Firstly, because of the cross-sectional design of the study it is not possible to infer any causal relationship. However, this type of non-experimental design is preferred when evaluating a novel target as it allows us to detect all the important correlations. A follow up to this study with an experimentally designed research would allow us to fully characterize any cause/effect relationships. Secondly, our focus on healthy young adults may have obscured associations found in studies utilizing unhealthy or older participants. Our population was formed by generally healthy, young, mostly white, well-educated participants that were normal to slightly overweight, thus, we cannot generalize our findings to other populations. Thirdly, while biologically and statistically significant effects were seen in the current study, it is possible that our sample size limited the strength of subgroup analysis, particularly when dividing the group by genders and extreme sleep groups. Future studies with larger numbers are strongly recommended to allow for a clearer representation of significant effects, as the effects found in the present study might represent only the strongest associations between our target variables. Although, the gender separation decreased the power of our analyses we deemed it necessary in order to prevent erroneous conclusions, given that sex distribution within sleep time was not uniform. Furthermore, we observed a significantly higher number of correlations in men than women (19 versus 6) and believe that hormonal variations in female participants might have resulted in increasing the standard deviation, obscuring our potential to detect associations in this gender. Therefore, we recommend future

research in the field to take into consideration the timing of the menstrual cycle when designing studies to analyze the gut microbiome in female participants. This suggestion is based on previous research showing substantial changes in food cravings and macronutrient composition, as well as sleep quality throughout the menstrual cycle (Baker et al. 2007; Gorczyca et al. 2016), factors that could play a role controlling the gut microbiome. Lastly, we strongly encourage researchers to try their best to run their samples in the least amount of batches or as one batch if possible, as we did observe a strong batch effect in our samples. Despite these limitations, the present study conserved several key strengths: 1) the objective measurement of body composition through DEXA, 2) the objective 7-day sleep and physical activity measurement through research-grade accelerometers, and 3) the meticulous selection of participants that helped to control for potential confounding variables affecting the microbiome as well as sleep patterns.

In conclusion, sleep quality and duration were not related with alpha diversity parameters in our young, healthy, normal BMI population with high physical activity levels.

We are only beginning to understand how microbial diversity contributes to the balance between health and disease, further research in the area will help us to better understand this novel area of research.

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