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Sleep problems are associated with changes in Firmicutes:Bacteroidetes ratio but not alpha and beta diversity

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SUMMARY Sleep is intimately tied to proper functioning of human physiology via a circadian rhythm. Accordingly, sleep problems are associated with negative changes leading to numerous diseases in humans (e.g. diabetes, cancer and hypertension), although it is unclear whether this is a causative or correlational relationship. Previous research has explored the relationship between sleep problems (e.g. insomnia) and the composition of the gut microbiota, with mixed findings on whether the gut microbiota changes in response to poor sleep. Our study aimed to further untangle the association between sleep problems and the human gut microbiota. Alpha diversity, beta diversity, and taxonomic analyses were performed on gut microbiota data of participants with and without self-defined sleep problems who were also part of a Parkinson's Disease study. QIIME2 and R were used for such analyses. We observed no difference in gut microbial diversity between subjects with sleep problems and healthy controls. Additionally, no differences between subjects with and without sleep problems were found based on the weighted UniFrac distances. A significant decrease in the Firmicutes:Bacteroidetes ratio was exhibited in individuals with sleep problems in contrast to those without sleep problems. Taken together, our findings do not fully reflect the results of past studies about differences in gut microbial diversity and Firmicutes:Bacteroidetes ratio, thus suggesting that the relationship between sleep problems and changes in the gut microbiota are more complex than previously thought.

INTRODUCTION

Sleep is an important biological process that is directly linked to multiple aspects of human physiology via a circadian rhythm, such as proper functioning of the immune, endocrine, and cardiovascular system (1, 2). Consequently, it has been found that the dysregulation of sleep and hence circadian rhythms will lead to significant negative consequences for human health. Notable conditions that negatively affect sleep quality include insomnia (an abnormal difficulty in falling asleep or staying asleep), sleep restriction (a reduction in sleep time below an individual's usual amount) and sleep deprivation (a complete elimination of sleep for a period of time) (3, 4). Serious morbidities associated with sleep problems in humans include diabetes, cancer, cardiovascular disease, and obesity (2). The brain and gastrointestinal system are intimately connected via the gut-brain axis, which can be defined as all afferent and efferent neural, endocrine, and nutrient signals between the central nervous system and the gastrointestinal system (5). Notably, the gut microbiota has emerged in recent years as a cornerstone of the gut-brain axis, especially since the gut microbiota and the brain have been found to communicate with each other through the immune system, the enteric nervous system, and even microbial metabolites such as short-chain fatty acids (6). Given the known pathophysiological role of sleep problems and the notable connection between the gut and the brain via the gut-brain axis, it naturally provokes the question of how sleep problems may contribute to changes in the gastrointestinal system and more specifically, the human gut microbiota.

Previous research has sought to characterize the relationship between sleep problems and the gut microbiota, albeit with mixed results. Several studies have found that the gut microbiota of people with sleep problems differs significantly from that of people without sleep problems (7–9). The composition, alpha diversity and metabolic function of the gut microbiota in people with insomnia was significantly changed compared to healthy controls

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(7). Notably, insomnia patients had Bacteroidetes as the dominant taxa in their gut microbiota, while Firmicutes and Proteobacteria were more prominent in healthy controls, resulting in a decreased Firmicutes:Bacteroidetes ratio (7). However, short-term sleep loss was found to alter human gut microbiota composition leading to an increased Firmicutes:Bacteroidetes ratio, in contrast to the ratio found in the previous study (8). These findings are noteworthy because Firmicutes and Bacteroidetes are regarded as the dominant bacterial phyla in the fecal microbiota of healthy adults (10). Regardless, both studies provided evidence that sleep problems do indeed affect the composition of the human gut microbiota. Finally, people who self-reported poorer sleep quality were found to have a less diverse gut microbiome with altered composition (9).

Due to the extensive biological similarities between mice and humans, murine models are commonly used for human gut microbiome research (11). Thus, the effects of sleep problems on the gut microbiome of mice has also been investigated to further characterize the sleep-gut microbiome relationship. Forced sleep deprivation for 5 hours in mice resulted in changes to the relative abundance of *Clostridiaceae* and *Lachnospiraceae*, although overall microbial composition did not change between the sleep-deprived and control mice (12). In a separate study, mice who were exposed to 5 days of repeated sleep disruption had altered beta diversity and an increased Firmicutes:Bacteroidetes ratio (13).

While the above studies support the notion that sleep problems are associated with changes to the gut microbiota, contradictory results have been reported where no differences were found in the composition of the gut microbiota of subjects with sleep problems compared to that of people without sleep problems. For example, humans who underwent artificially induced sleep restriction in a laboratory setting exhibited no significant changes in gut microbial richness or composition, with no changes to the Firmicutes:Bacteroidetes ratio (14). As a result, although the majority of studies demonstrate changes in the gut microbiota due to poor sleep, the correlation between sleep problems and gut microbiota is still inconclusive and necessitates additional investigation.

After analyzing our data, we report no difference in gut microbial evenness and richness between subjects with and without sleep problems. We also observed no difference in gut microbial composition between subjects with sleep problems and those unaffected by sleep problems, contradicting some of the findings of previous literature. However, we found a significant decrease in the Firmicutes:Bacteroidetes ratio in people with sleep problems, which provides evidence of a link between sleep and the abundance of these two phyla in the gut microbiome. Our results provide evidence that the association between sleep problems and the gut microbiota may be more complicated than previously believed, where different kinds of sleep problems may affect the gut microbiota in different ways.

METHODS AND MATERIALS

All QIIME2 and R scripts, including any unspecified parameters, are included in the supplementary text files.

Study cohort and data collection. Study participants' data were taken from a previously published study regarding the impact of Parkinson's Disease (PD) on the gut microbiota (15). Various data were collected from the participants, including (but not limited to) diet, sex, medication and sleep problems (15). The presence or absence of sleep problems were self-reported by participants (15). Our study only examined the data from healthy controls (no PD patients) that had reported the presence or absence of sleep problems.

Gut microbiota sequence analysis. Sequence quality control, which includes truncation of amplicon sequence variants (ASVs) to a length of 251, was done using DADA2 (16). Patients with Parkinson's Disease, participants who had no recorded data for sleep problems, chloroplast, mitochondria, archaea, and low frequency ASVs (feature frequency <55) were filtered out with QIIME2 (v 2020.8) (17). Samples were rarified to a sampling depth of 10403.

Alpha diversity analyses of subjects' gut microbiota. For a greater understanding on the indeterminate changes in gut microbial richness and evenness with sleep problems, Pielou's

evenness & Observed features plots were completed using QIIME2 and its significance was tested with Kruskal-Wallis (7–9).

Beta diversity analyses of subjects' gut microbiota. In order to better ascertain the inconclusive compositional differences of the gut microbiota with the occurrence of sleep problems, weighted UniFrac Principal Coordinates Analysis (PCoA) was generated using R (v 1.3.1093) (7–9, 12–14, 25). Weighted UniFrac box plots were also conducted using QIIME2 and its significance was tested with PERMANOVA. Separate analyses were run to see how antidepressant use, gender and coffee consumption may have respectively impacted the gut microbiota.

Taxonomic analysis of subjects' gut microbiota. To identify predominant taxa between individuals with and without sleep problems, taxonomic bar plots were generated using QIIME2. Most notably, we wanted to see whether the Firmicutes and Bacteroidetes phyla remain the most dominant taxa of the gut microbiota, as previous literature suggested (7, 8, 14).

Differential abundance analysis of subjects' gut microbiota. In order to determine whether there is a change in the abundance of certain taxa with the presence of sleep problems, we conducted differential abundance analysis at the family level. We further investigated the relative abundance of Firmicutes and Bacteroidetes, which has been previously shown to differ with the presence of sleep problems (7, 8, 13, 14). The log (base 2) ratio of Firmicutes to Bacteroidetes was utilized to analyze this relationship and its significance was tested with ANOVA.

RESULTS

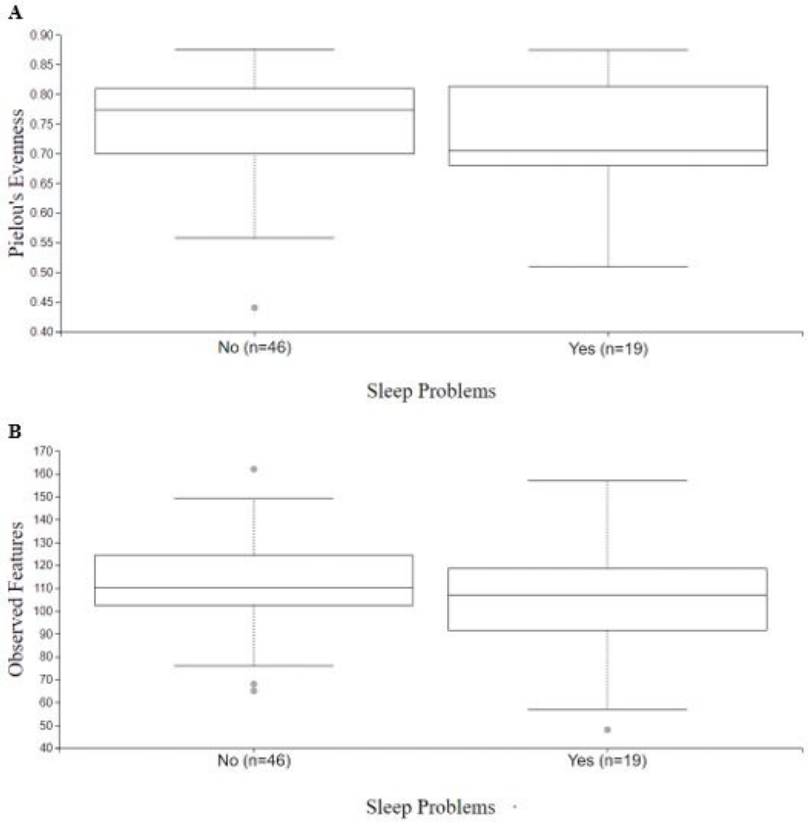

FIG. 1 Gut microbial evenness and richness do not differ between subjects with and without sleep problems. The right and left boxes depict subjects with ('Yes') and without ('No') sleep problems, respectively. Sample size is 46 for the 'No' sleep problems groups and 19 for 'Yes' sleep problems groups. Median values are indicated by the horizontal line in-between each box. **(A)** Pielou's Evenness box plot for sleep problems indicates a median Pielou's Evenness value of 0.77 and 0.70 for the 'No' and 'Yes' sleep problems groups, respectively (Kruskal-Wallis, p=0.3948). Pielou's Evenness value reflects the gut microbial evenness. **(B)** Observed features box plot for sleep problems showcases a median observed features value of 110 and 105 for the 'No' and 'Yes' sleep problems groups, respectively (Kruskal-Wallis, p=0.3337). Observed features value indicates the number of ASVs in the gut microbiota, also known as gut microbial richness.

Sleep problems are not associated with changes in gut microbiota evenness and richness. Previous research showed inconclusive results regarding the association between sleep problems and gut microbial evenness and richness (7, 14). In order to test whether sleep problems are associated with changes in gut microbial evenness and richness, we calculated Pielou's Evenness and microbial richness for individuals with and without sleep problems

(Fig. 1). We found a slight drop in the median microbial evenness from 0.77 to 0.70 with the occurrence of sleep problems (Fig. 1A), however, the change was found to be nonsignificant (Kruskal-Wallis, p=0.3948). Similarly, we found a relatively negligible change in the median richness from those without (110) and with (105) sleep problem (Kruskal-Wallis, p=0.3337, Fig. 1B).

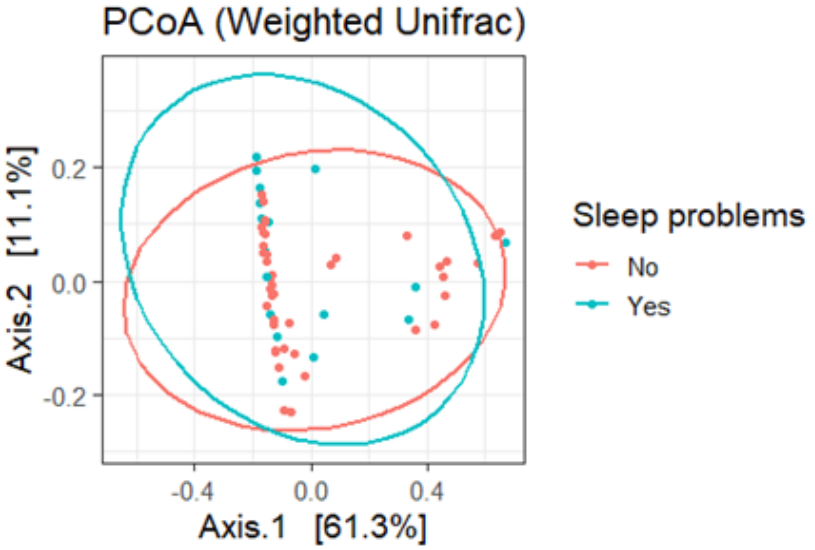

FIG. 2 Gut microbial composition does not differ between subjects with and without sleep problems. Weighted Unifrac distances were measured between samples with ('Yes') and without ('No) sleep problems. 'n' represents the number of combinations of samples within each group. Sample size is 46 for subjects without sleep problems and 19 for subjects with sleep problems. Two axes of weighted Unifrac PCoA plot accounts for 72.4 percent variance. Each data point represents the microbial composition of a sample. Turquoise represents subjects with sleep problems and orange represents subjects without sleep problems. Ellipses represent clustering of samples from each group based on distribution of the samples.

Sleep problems are not associated with compositional change in the gut microbiota. To determine whether sleep problems correspond to a change in gut microbial composition, we conducted beta diversity analysis based on weighted UniFrac distances. The weighted UniFrac PCoA plot showed no obvious clustering for those with or without sleep problems (Fig. 2). The lack of clustering or distinct pattern that differentiates those with and without sleep problems suggests that there is no difference in the gut microbial composition between subjects with and without sleep problems. Median pairwise weighted UniFrac distances were found when the microbiota of those without sleep problems were compared to each other (0.26) and those with sleep problems (0.30), as well as when the microbiomes of those with sleep problems were compared (0.29) (PERMANOVA, p=0.211, Fig. 3). This indicates that the difference is not statistically significant and that sleep problems are not correlated with a difference in gut microbial composition.

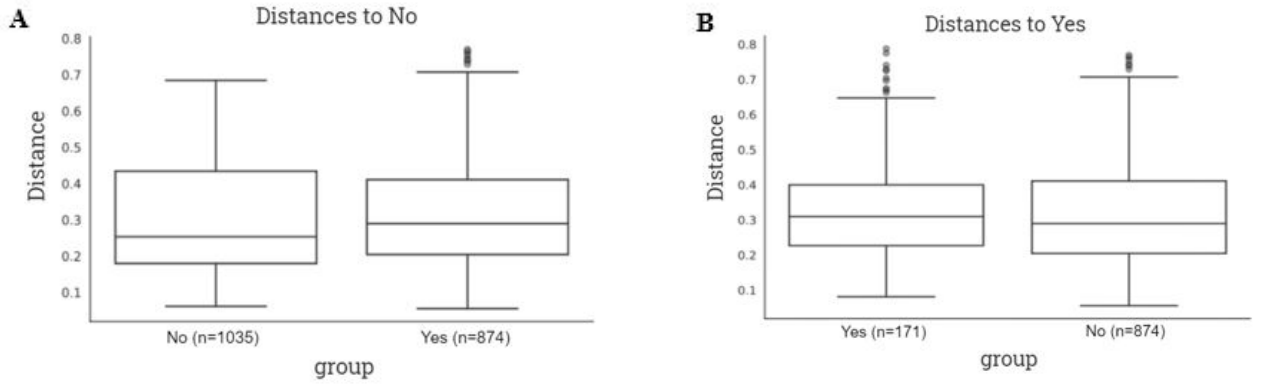

FIG. 3 No statistically significant difference in gut microbial composition associated with sleep problems. (A) Using a subject without sleep problems ('No') as the basis for comparison, participants without sleep problems had a median distance value of 0.26 while those with sleep problems had a value of 0.30. Subjects without sleep problems are shown on the left-hand side ('No'), and subjects with sleep problems are shown on the right-hand side ('Yes'). n =1035 for those without sleep problems and n = 874 for those with sleep problems. (B) Using a subject with sleep problems as the basis for comparison, participants without sleep problems had a median distance value of 0.29 while those with sleep problems had a value of 0.3. Subjects without sleep problems are shown on the right-hand side, and subjects with sleep problems are shown on the left-hand side. n = 874 for those without sleep problems and n = 171 for those with sleep problems. Significance was tested with PERMANOVA, p = 0.211.

Sleep problems are associated with a decrease in the Firmicutes to Bacteroidetes ratio of the gut microbiome. Conflicting results about the association of sleep problems on Firmicutes to Bacteroidetes ratio in the gut microbiome exist (7, 8). To determine whether there is a change in the ratio of Firmicutes to Bacteroidetes, we conducted taxonomic analysis. While differential abundance analysis was run to test for changes in the relative abundance of specific phyla, family and species between those with and without sleep problems, no significant changes in relative abundance were found. At the Phylum level, regardless of the presence of sleep problems, the gut microbiota was heavily dominated by Firmicutes and Bacteroidetes (Fig. 4). An increase in the relative abundance of Bacteroidetes (0.38 → 0.46) and a decrease in the relative abundance of Firmicutes (0.52 → 0.44) was found with the occurrence of sleep problems (Fig. 5B). These changes in the relative abundance of Firmicutes and Bacteroidetes led to a significant decrease in the ratio for the relative abundance of Firmicutes to Bacteroidetes for those with compared to without sleep problems (~1.1 → 1, p=0.0292) (Fig. 5A). These results indicate that while both Firmicutes and Bacteroidetes remain the most dominant phyla of the gut microbiota regardless of sleep problems, sleep problems are associated with a significant increase in the relative abundance of Bacteroidetes, and a decrease in the Firmicutes to Bacteroidetes ratio.

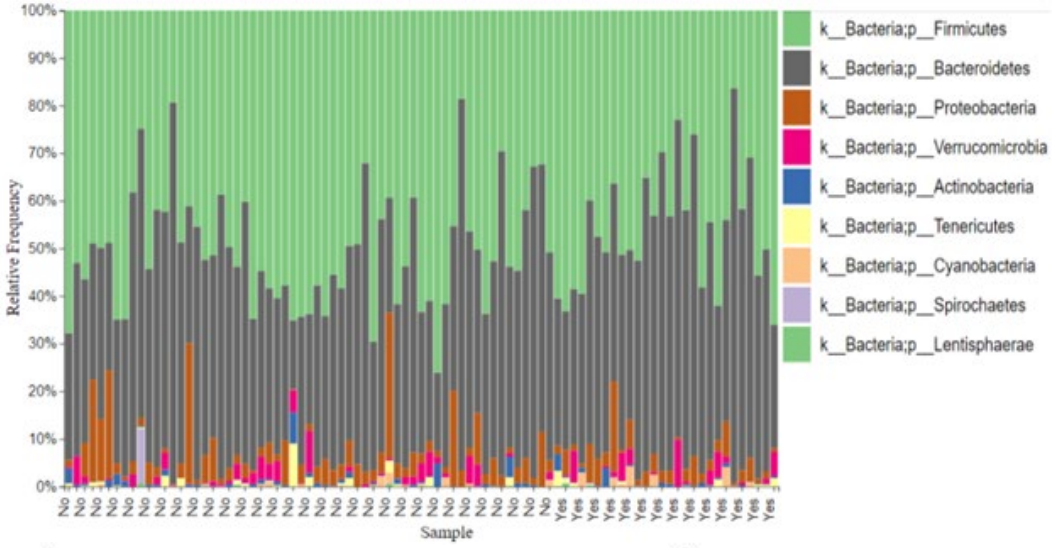

FIG. 4 Firmicutes and Bacteroidetes are the most dominant gut microbiota phyla. **(A)** Taxa bar plot for taxa level 2 (phylum) showing Firmicutes in green, Bacteroidetes in grey and other phyla color coded in order of abundance on the right-hand side.

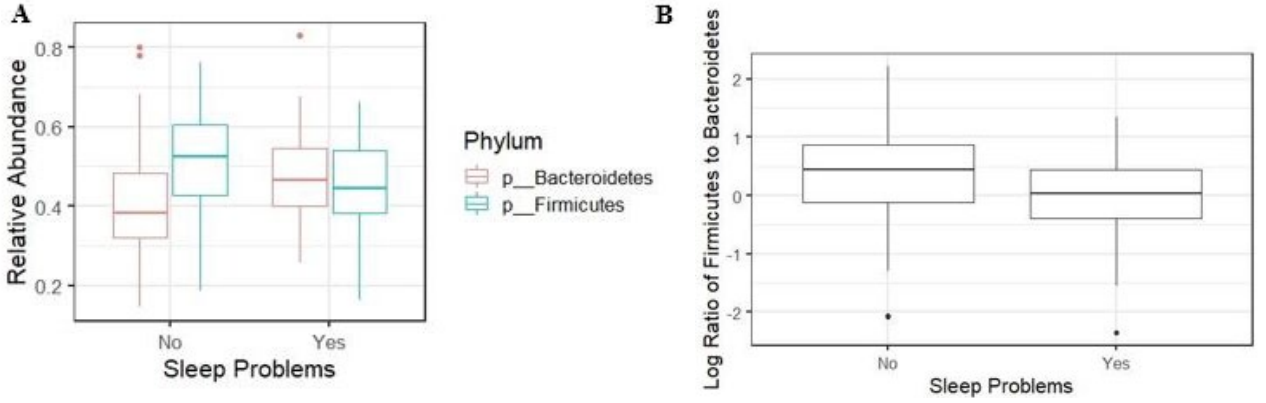

FIG. 5 Sleep problems are associated with a decrease in Firmicutes to Bacteroidetes ratio. (A) Relative abundance box plot of Bacteroidetes (orange) and Firmicutes (turquoise) for subjects without ('No') and with ('Yes') sleep problems. Subjects without sleep problems had a median relative abundance for Bacteroidetes and Firmicutes of 0.38 and 0.52, respectively. Subjects with sleep problems had a median relative abundance for Bacteroidetes and Firmicutes of 0.46 and 0.44, respectively. (Pairwise ANOVA for Bacteroidetes, p=0.0228; Pairwise ANOVA for Firmicutes, p=0.0514). (B) Box plot of the logarithmic ratio for the relative abundance of Firmicutes to Bacteroidetes in subjects without sleep problems ('No') are shown on the left-hand side and those with sleep problems ('Yes') are presented on the right-hand side. Logarithmic base 2 ratio is 0.136 in the "No" sleep problems group and ~0 in the "Yes" sleep problems group (ANOVA, p=0.0292). The non-logarithmic ratio is ~ 1.1 and 1, respectively.

DISCUSSION

Prior studies that have seen a correlation between sleep problems and increased changes in gut microbiota diversity have primarily studied healthy young adults (18, 19). Our study collected data from British Columbian seniors with a mean age of 64, who were age-matched controls in a separate study investigating the effect of Parkinson's Disease on the gut microbiota (15). Therefore, this study sheds light on the association between sleep problems and gut microbiota in a population that was not previously studied. Since our study showed no significant differences in gut microbiota between those with and without sleep problems, which is opposite to the results from previous studies using younger cohorts, there could be other variables in older populations that are overshadowing the effect of sleep problems on gut microbiota. It has been shown that aging is associated with an increase in the gut microbial diversity (20, 21). It is possible that the increase in the diversity of the gut microbiota observed in older individuals means that the effect of sleep problems on gut microbiota is potentially less pronounced.

Another probable reason why our study showed different results compared to previous studies is that there could be an extraneous variable that overshadows the effect of sleep problems on gut microbiota. This is further supported by our investigation of variables such as antidepressant use, gender and coffee consumption, which have been previously shown to be associated with significant changes in the gut microbiota (22–24). However, our analysis found no differences in gut microbiota composition correlated with variables relevant to gut microbiota diversity like antidepressant use, gender, and coffee consumption (Fig. S1-3). Therefore, these contrasting results suggest the presence of an extraneous variable. Variations in diet have been found to have the largest effects on the composition of the adult gut microbiota compared to other factors (19). As we did not control for diet, it is possible that differences in dietary intake among our participants were a stronger determinant of gut microbiota diversity and confounded our analysis on the impact of sleep problems, antidepressant use, gender, and coffee consumption on the gut microbiota.

Similar to our results, a previous study showed a change in the Firmicutes to Bacteroidetes ratio despite no change in overall gut microbial diversity (8). This suggests that the observed decrease in the Firmicutes:Bacteroidetes is more sensitive to change than gut microbial composition, richness, and evenness. We postulate that this could be due to the fact that the change in relative abundance of the phyla wasn't large enough to cause a significant difference in the overall gut microbial diversity.

The relationship between sleep and the gut microbiota has been analyzed in the context of a wide array of sleep-related problems including sleep disorders, sleep deprivation and sleep restriction (7, 12, 14). Significant changes in the beta diversity of the gut microbiota have been observed in individuals suffering from insomnia as well as mice with short-term sleep deprivation (7, 12). In contrast, sleep restriction has not been shown to be associated with the gut microbiota in either humans or mice (14). Furthermore, different changes in the Firmicutes to Bacteroidetes ratio in the gut have been shown to be associated with the type of sleep problems analyzed (7, 8, 14). Partial sleep deprivation and insomnia has been shown to cause changes in this ratio, while sleep restriction did not (7, 12, 14). Sleep restriction is defined as a reduction in sleep time below an individual's usual amount (4). In contrast, sleep deprivation is defined as a complete elimination of sleep for a period of time and insomnia is defined as an abnormal difficulty in falling asleep or staying asleep (3, 4). By definition, people undergoing sleep restriction are able to sleep more and have higher quality sleep compared to individuals experiencing sleep deprivation or insomnia. As a result, it is possible that the differing durations of sleep across the aforementioned sleep conditions may explain why contrasting results were observed.

Limitations As sleep problems were subjectively defined by the participants in our study, it is unknown whether participants suffered from a clinically verified sleep disorder, sleep deprivation, or sleep restriction. Additionally, the length and severity of the sleep problems experienced were not reported by participants, even though both have been shown to be factors that affect how the gut microbiota changes in response to sleep problems (8, 14, 18). Long-term sleep loss has been shown to cause more significant changes in the gut microbiota composition compared to short-term sleep loss (8, 14, 18). In addition, increasing amounts of

sleep disruption have been found to correlate with increasingly significant changes in gut microbiota composition and taxonomic profile (18). Without knowing the nature, extent, and severity of sleep problems experienced by participants, it is difficult to appropriately characterize the effects of sleep on the gut microbiota composition and diversity. This may explain why changes in gut microbiota composition were not observed.

Conclusions Our study mainly contradicted the findings of previous studies that investigated the relationship between sleep problems and the gut microbiota. No differences in gut microbial evenness and richness were found between subjects with and without sleep problems. Additionally, no differences in gut microbial composition were exhibited between individuals that do and do not experience sleep problems. A significant decrease in the Firmicutes:Bacteroidetes ratio and an increase in the relative abundance of Bacteroidetes were observed in people with sleep problems. Based on our findings in the geriatric population, sleep problems are not associated with a change in the gut microbial composition, richness, and evenness, but is correlated with an increase in the relative abundance of Bacteroidetes and a decrease in the Firmicutes to Bacteroidetes ratio. Taken together, our study and previous literature leave open the possibility that diet and distinct types of sleep problems can have significant implications on the gut microbiota.

Future Directions While our study did not find significant differences in gut microbiota diversity between people who have sleep problems and those that do not, we could optimize our study design in future investigations to better isolate the effect of sleep problems. Specifically, future studies should incorporate samples from a variety of geographical regions to account for the potential effect of geography on the human gut microbiota. Additionally, diet should be controlled since it is a well-known variable affecting the gut microbiota. Since we mainly looked at a geriatric population, it would also be worthwhile to investigate the effect of sleep problems on the gut microbiota of a younger population. We have noted the possibility that different sleep disorders may change the gut microbiota in different ways since they vary in the amount of sleep that an individual is receiving. Thus, it may not be sufficient to simply look at sleep problems as a categorical variable. Instead, we could record the self-reported amount of time that subjects have been sleeping (if they have been able to sleep at all) in order to add a quantitative aspect to our analysis.

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