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The Component and Functional Pathways of Gut Microbiota Are Altered in Populations with Poor Sleep Quality – A Preliminary Report

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Abstract

With the development of genome sequencing, many researchers have investigated the mechanism by which the intestinal microbiota influences sleep across the brain-gut axis. However, the relationship between gut microbiota and sleep disorder remains unclear. Thus, we studied the difference in gut microbiota composition between poor sleep quality- and normal populations, which helps set the ground for future research. The recruited college students provided baseline information and stool samples and completed the Pittsburgh Sleep Quality Index (PSQI). We compared the two groups' gut microbiota composition and functional differentiation by using the 16S rRNA gene sequencing analysis. The main bacterial difference and the most critical effect were mainly concentrated within Tenericutes and Elusimicrobia. Compared with the healthy control group, some functions of the gut microbiota were impaired in the poor sleep quality group, such as butanoate metabolism and propanoate metabolism. Bacterial taxa with significant differences raised the possibility for future diagnosis and treatment of sleep problems.

K e y w o r d s: sleep disorder, poor sleep quality, gut microbiota, 16S rRNA, college students

Introduction

A sleep disorder (SD) is characterized by difficulty in falling asleep, lack of sleep, excessive sleep, and poor sleep quality, which can affect the personal physical status and inherent mental health. Overwhelming evidence indicates that sleep plays an irreplaceable role in protecting human health, eliminating fatigue, restoring body function, enhancing immunity, protecting brain health, and others (Irwin 2015). However, previous studies show that inadequate sleep was found in 40 ~ 65% of American students (Carney et al. 2006; Lund et al. 2010; Kenney et al. 2012; Becker et al. 2014). Correspondingly, according to a recent study investigating 11,954 students from 50 universities in China, relatively severe sleep disorders were found among students attending high-level universities (Yang et al. 2018). These studies also suggested that academic and job strain and adverse lifestyle habits may be the main reason for sleep problems. One study of 1,125 participants from four countries provided novel evidence for the deterioration of problematic sleep among adolescents that at least one insomnia symptom was reported by 32.7% of adolescent workers and 35.2% of non-employed adolescents. Additionally, this study also indicated that insomnia symptoms were persistent in individuals with anxiety (73.2 ~ 76.5%) or depression (67.6 ~ 77.4%) disorders (Ohayon et al. 2000), which suggested that comorbidity led to the development and worsening of sleep disorder to a certain extent. Given the high prevalence of sleep problems in adolescents and the expanded influence on health outcomes, it has become a severe public health concern, which is needed to be solved urgently.

Recent studies have generally discovered the linkages between gut microbiota and sleep disturbance with

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the development of genome sequencing techniques. There is currently some evidence in the mice model that sleep disturbance can alter the composition of the gut microbiome (Poroyko et al. 2016). Subsequently, some findings that fecal microbiota transplantation could result in sleep disturbances supported the bidirectional linkages between the two (Badran et al. 2020). Additionally, a significant change in some bacterial taxa was reported by recent studies related to sleep, such as an increase in the Firmicutes to Bacteroidetes ratio (Bowers et al. 2020) and an alteration of the relative abundance of the *Clostridiaceae* and *Lachnospiraceae* (El Aidy et al. 2020) in sleep-disrupted mice. It might highlight possible directions of future research in this field. Although most mice experiments can consistently reveal the association between sleep problems and gut microbiota, some studies in humans have reported contradicting conclusions (Benedict et al. 2016; Zhang et al. 2017), suggesting that previous works needed to be repeated to verify the question. Another concern is that antibiotics and dietary prebiotics have been utilized to alter the gut microbiota and, correspondingly that sleep quality was influenced by the changes in the gut bacteria (Nakakita et al. 2016; Thompson et al. 2017). In short, the findings of previous studies, both reduction in nocturnal sleep (Lendrum et al. 2017) and metabolic disturbance of amino acids and vitamins related to neurotransmission (Ogawa et al. 2020), have been strengthened the association between gut microbiota and sleep disturbance by using antibiotics to deplete the gut microbiota of mice. The research on dietary prebiotics used to improve sleep is in its infancy, and there are still many issues to be elucidated, including safety, efficacy, and others. Even though the causal associations between the gut microbiome and sleep problems remain undetermined, the above findings indicated that gut microbiota is essential for improving sleep quality.

To this end, we conducted a study in which the differences in the diversity and composition of the gut microbiota between the individuals with poor subjective sleep quality and normal were analyzed. We tried to explore possible alteration of functional pathways and identify the significant gut bacteria for further studies.

Experimental

Materials and Methods

Participants. This study was approved by the Institutional Examination Board of the Anhui Medical University. All subjects were volunteers recruited among university students. To ensure the comparability between the two groups and reduce the confounding bias, the enrolled participants were screened by the exclusion criteria. Subsequently, they were divided into groups of healthy control (HC) and sleep disorder (SD) based on the good and poor subjective sleep quality. Finally, 34 individuals were included for further analysis (each group has 17 individuals). Subjects' data is shown in Table I. Exclusion criteria included a history of the brain, neurological, psychiatric disease, history of alcohol dependence, and using any antibiotic or supplements within the last 30 days. Participants reported not suffering from any gastrointestinal disorder, and none of the subjects recently suffered from severe diarrhea and constipated disease. No one had a special diet habit during the previous two years. They kept a regular diet for the past week. All participants signed the written informed consent form.

Collection of demographic and sleep condition information. All participants underwent the questionnaires, which included gender, age, BMI (Body Mass Index), and the Pittsburgh Sleep Quality Index (PSQI) score (Buysse et al. 1989). The PSQI is a self-report questionnaire consisting of 19 items and five additional questions, which was widely used to assess subjects' sleep quality over the past month. It consists of seven domains: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbance, sleep medication use, and daytime dysfunction. Total-scores range from 0 to 21, and higher scores mean poorer sleep quality. Previous studies (Guo et al. 2016) suggested that the Chinese version of PSQI, with a cutoff of 8, showed good reliability in Chinese people. In the current study, the subjects whose PSQI global score >7 are defined as having poor sleep quality and would be assigned to the SD group, if not to the HC group.

Table I
Demographic characteristics of the participants.

Characteristics	SD (n = 17)	HC (n = 17)	t/χ^2	*p*-value
Male/Female	10/7	7/10	0.159	0.303
Age (years)	20.059 ± 0.785	19.662 ± 0.611	1.646	0.109
BMI (kg/m^2)	21.229 ± 2.194	20.167 ± 2.172	1.419	0.166
PSQI score	9.059 ± 1.144	5.118 ± 1.576	8.342	< 0.001

p-value < 0.05 are considered statistically significant

Collection of fecal samples. Each participant was instructed to collect fecal samples through the correct approach between 07:00 and 09:00 of the following day with the sterile stool collection kit and as quickly as possible to arrive at the laboratory. The partial sample of the middle, the inner fragment of the fecal, was placed in a centrifuge tube by the operator using a sterile spoon, which finished on the ultraclean table after exposure to ultraviolet irradiation. Finally, these centrifuge tubes and all samples were stored at –80°C until further analyses.

Genomic DNA extraction. Total bacterial DNA was extracted by operators using the QIAamp DNA stool Mini Kit (Qiagen, Germany). The NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was utilized to measure the DNA content and purity, and the agarose gel electrophoresis evaluated its integrity.

High-Throughput Sequencing. The V3-V4 hypervariable region of the 16S rRNA gene was amplified with the forward primer (5'-CCTACGGGNGGCWGCAG-3') and reverse primer (5'-GACTACHVGGGTATCTAATCC-3'). PCR amplification products were detected using agarose gel electrophoresis and purified by the AgencourtAMpure XP kit (Beckman Coulter, USA). The 16S rRNA gene amplification products sequencing was performed with the 2 × 250 bp paired-end method using the Illumina MiSeq Benchtop Sequencer (Kozich et al. 2013) at the Genesky Biotechnologies Inc. (Shanghai, China). The 16S rRNA amplicon sequences were deposited in Sequence Read Archive (SRA) database with the accession number (BioProject ID: PRJNA788172). The SRA is accessible at https://dataview.ncbi.nlm.nih.gov/object/PRJNA788172.

Bioinformatics analysis. To ensure the accuracy and reliability of bioinformatics analysis results, the raw reads were filtered by the QIIME2 to remove low-quality and polyclonal sequences and then merged by using FLASH (Magoč and Salzberg 2011). Primers in sequences were checked and removed by Mothur software. The research software was utilized for removing the sequences with a total base error rate > 2 and length less than 100 bp to obtain the clean reads with high quality and reliability. The clean reads were clustered into operational taxonomic units (OTUs) based on a 97% similarity cutoff by the UPARSE pipeline (Dong et al. 2017) after removing chimeras and singletons.

All OTUs were classified based on the Ribosomal Database Project (RDP) database (version 11.5), and the microbial community abundance and diversity were calculated by alpha-diversity indices, including observed species, Chao1, and ACE for microbial richness, and the Shannon index, Simpson index, and Coverage index for microbial diversity. Beta-diversity was calculated to determine the difference between the SD and HC groups, including Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance, Jaccard, and unweighted and weighted uniFrac metrics. Moreover, the ADONIS analysis with a nonparametric implementation of a Permutational Multivariate Analysis of Variance (PERMANOVA) and the Partial Least Squares Discriminant Analysis (PLS-DA) that has a better effect when the difference between groups is not significant was used. The metastats analysis was used to identify the changes in microbiota composition. The linear discriminant analysis (LDA) effect size (LEfSe) was performed to detect the difference in species abundance between two groups, and further, the species were obtained with a significant difference. LDA analysis was used to estimate the impact of these species on the differences between the two groups. The larger the LDA score, the greater the influence of species abundance on the difference effect, and only species with an LDA score > 2.0 were kept (p-value < 0.05). Functional pathways of the identified taxa with differential abundance were inferred from OTUs using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved State (PICRUSt) and annotated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Langille et al. 2013).

Statistical analysis. In the present study, demographic data were analyzed by SPSS 22.0. Categorical variables were compared by Pearson's chi-square test, and Student's t-test performed quantitative variables. The Kruskal-Wallis test was utilized to analyze the relative abundances (%) of the gut bacterial communities and ADONIS analysis was used to determine the differences between the two groups, and whether the differences were statistically significant. PCoA and PLS-DA were carried out using packages of the R software (v4.0.3). Correlations were analyzed using Spearman correlation and plotted by the ggplot2 package in R software.

Results

The Basic Characteristics of the SD and HC Groups. Demographic characteristics of the SD and HC groups are presented in Table I. The PSQI score of the two groups were 5.118 ± 1.576 (HC) and 9.059 ± 1.144 (SD), with a significant difference (t = 8.342, p-value < 0.001). However, there was no significant difference in gender, age, and BMI. Both groups had similar demographic profiles.

The Quality of Fecal Samples. Rarefaction curve, Shannon-Wiener curve, rank-abundance curve, and species accumulation curve were utilized to assess the quality of the fecal samples, demonstrating that the fecal samples collected from all participants meet the requirements for experimental design and subsequent analysis (see supplementary materials).

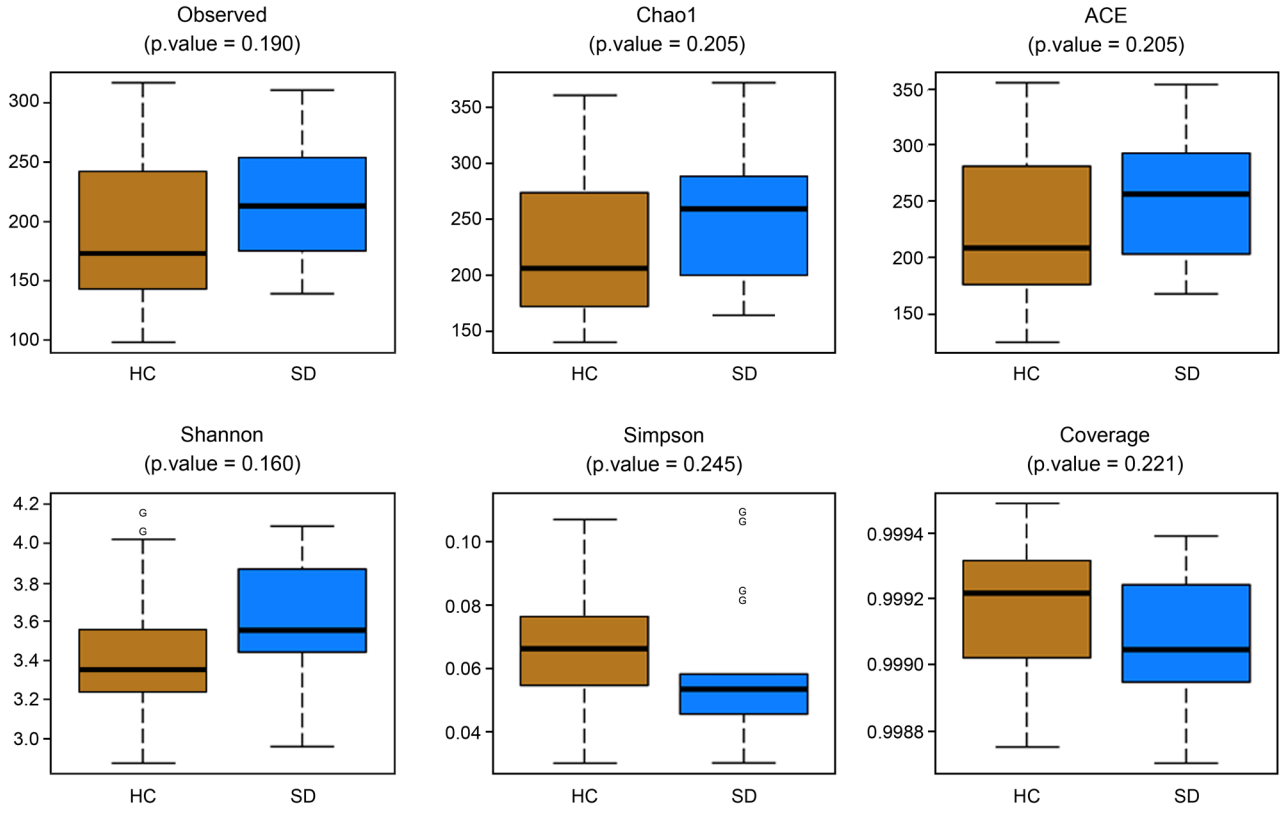

Fig. 1. Comparison of gut microbiota's alpha diversity indices between the SD and HC groups, including Observed, Chao 1, ACE, Shannon, Simpson, and Coverage. Plotted in the graphics are the interquartile ranges and boxes, medians (lines in the box), and the lowest and highest values for the first and third quartiles. The abscissa represents a different group; the ordinate represents the value of each diversity index. Different colors distinguish different groups.

Alpha diversity analysis. The six parameters above were compared based on the Wilcoxon rank-sum test of both groups to identify differences in alpha diversity between the SD and HC groups. Neither the species richness (observed species, Chao1 and ACE) nor diversity (Shannon index, Simpson index, and Coverage index) found significant differences between the two groups (all p-value > 0.05, two-side; Fig. 1).

Beta diversity analysis. We calculated unweighted UniFrac, weighted UniFrac, Jaccard, and Bray-Curtis distances of the microbial composition to determine differences in the bacterial community structure among samples from two groups in this study. ADONIS results revealed no significant differences between Jaccard (p-value = 0.1838, Fig. 2A) and Bray-Curtis distances (p-value = 0.5401, Fig. 2B). Therefore, we utilized the

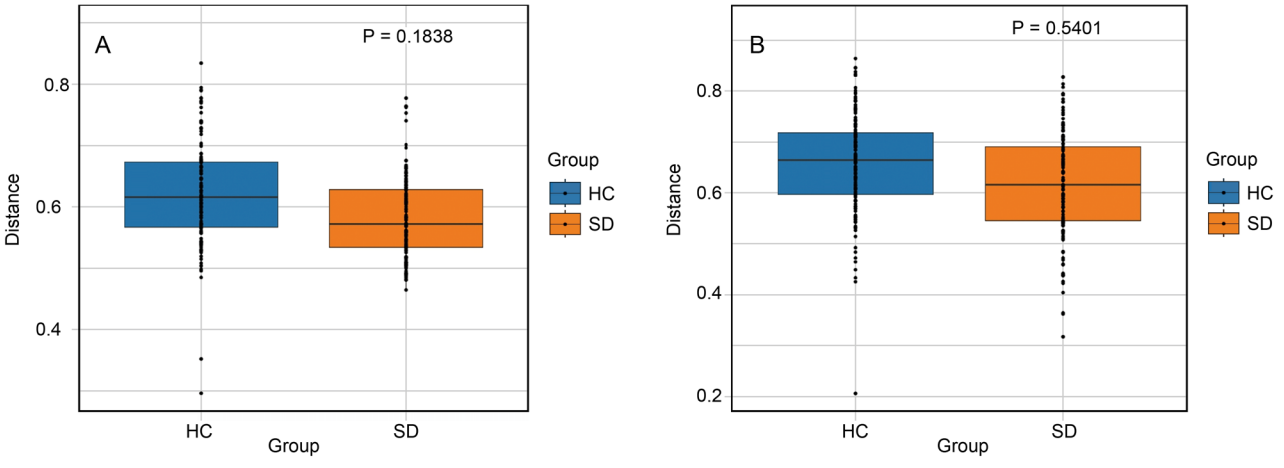

Fig. 2. The boxplot chart shows the beta diversity of the bacterial communities from the two groups, based on the Jaccard (A) and Bray-Curtis (B) distances, respectively. The interquartile ranges and boxes, medians (lines in the box), and the lowest and highest values for the first and third quartiles are plotted in the graph. Colors identify each group, and a black dot represents each sample.

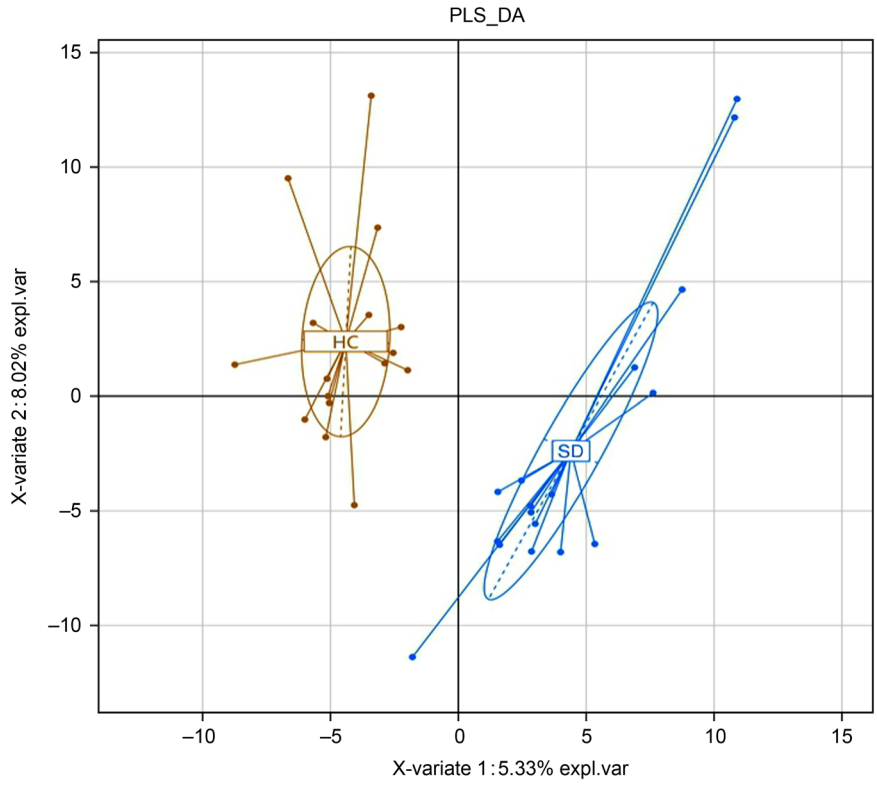

Fig. 3. Different color dots represent different groups; the horizontal and vertical axis scale is the relative distance without a practical significance; X-variable 1 and variable 2 represent the putative factors influencing changes in the microbial composition of two groups of samples, respectively. The plot is based on the weighted Unifrac distance.

PLS-DA, a supervision pattern recognition method, to assess the subtle significant differences between the two groups, and the result demonstrated that the two groups were separated obviously, as can be seen from Fig. 3.

Differential microbiota analysis. The linear discriminant analysis (LDA) and effect size (LEfSe) analysis, based on the nonparametric factorial Kruskal-Wallis (KW) sum rank test and Wilcoxon rank-sum test, were utilized to examine bacterial taxa with significant differential abundances between the SD and HC groups (LDA score > 2.0, p-value < 0.05, two-side). The result of LEfSe analysis showed 24 taxa with significantly different abundance, mainly focused on phyla Firmicutes and Proteobacteria, and the top ten species with the smallest p-value were shown in Fig. 4. The bacterial species with significant differences in relative abundance at each taxonomic level between the two groups were defined by the Metastats analysis under p-value < 0.05. There were eight taxa with significantly different abundance between the two groups after Bonferroni-Holm

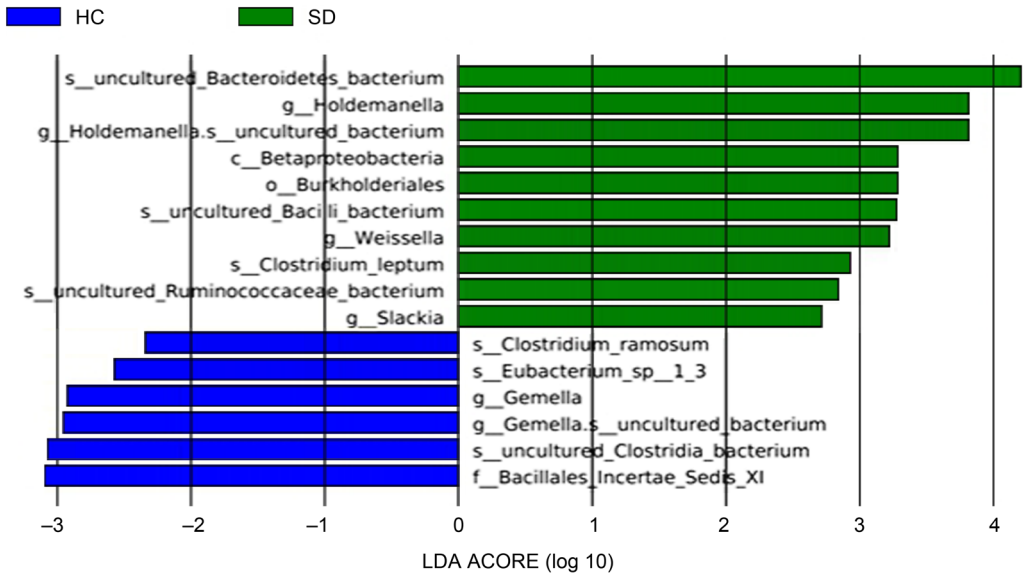

Fig. 4. The bacterial taxa's linear discriminant analysis (LDA) effect size (LEfSe). LEfSe plot shows the top ten species with the smallest p-value for each group. Ten bacterial taxa were enriched in the SD group (green), and six bacterial taxa were enriched in the HC group (blue).

Table II
Correlation analysis of the selected bacterial species with the PSQI score.

Level	Taxon name	Relative abundance (%)	*q*-value	*r*
Phylum	Elusimicrobia	0.171 ± 0.995	0.007*	–0.108
Phylum	Tenericutes	0.123 ± 0.631	0.030*	–0.183
Class	Elusimicrobia	0.171 ± 0.995	0.023*	–0.108
Class	Mollicutes	0.123 ± 0.631	0.069	–0.183
Class	Erysipelotrichia	2.606 ± 2.604	0.276	0.384*
Order	Elusimicrobiales	0.171 ± 0.995	0.014*	–0.108
Order	Anaeroplasmatales	0.123 ± 0.631	0.058	–0.183
Order	Desulfovibrionales	0.303 ± 0.259	0.188	0.328
Order	Erysipelotrichales	2.606 ± 2.604	0.208	0.384
Family	*Elusimicrobiaceae*	0.171 ± 0.995	0.027*	–0.108
Family	*Anaeroplasmataceae*	0.123 ± 0.631	0.110	–0.183
Family	*Desulfovibrionaceae*	0.303 ± 0.259	0.286	0.328
Family	*Erysipelotrichaceae*	2.606 ± 2.604	0.339	0.384*
Genus	*Holdemanell*a	0.647 ± 1.490	0.033*	0.601**
Genus	*Elusimicrobium*	0.171 ± 0.995	0.033*	–0.108
Genus	*Asteroleplasma*	0.123 ± 0.631	0.159	–0.183
Species	*uncultured_proteobacterium*	0.128 ± 0.748	0.035*	–0.183
Species	*uncultured_bacterium*	3.700 ± 2.810	0.707	0.323

q-value BH-corrected; * – *q*-value < 0.05; ** – *q*-value < 0.01

(BH) correction (*q*-value < 0.05). Additionally, the result of the Spearman correlation indicated that only three taxa had a significant positive correlation with PSQI score, respectively, and the correlation coefficient for genus *Holdemanella* reached its maximum value ($r = 0.601$, p-value < 0.01) (Table II). The actual correlations between them and the SD score were shown with scatter plots in Fig. 5.

Functional prediction of gut microbiota. As our results indicated a shift in the gut microbiome composition, we further examined whether it might relate to potential metabolic differences between the SD and HC groups, as previous studies have shown. The functional metagenome (PICRUSt) was utilized for characterizing the functions of the gut microbiota. Subsequently, Welch's *t*-test was used to identify statistically significant differences between the two groups (Fig. 6). The results suggested that compared to the HC group, metabolic pathways involved in polycyclic aromatic hydrocarbons pathways degradation (p-value = 0.005, two-side) were significantly increased in the SD group. In contrast, metabolic pathways involved in styrene degradation (p-value = 0.009, two-side), butanoate metabolism (p-value = 0.018, two-side), C5-branched dibasic acid metabolism (p-value = 0.023, two-side), propanoate metabolism (p-value = 0.043, two-side) and beta-alanine metabolism (p-value = 0.044, two-side) were significantly decreased in the SD group.

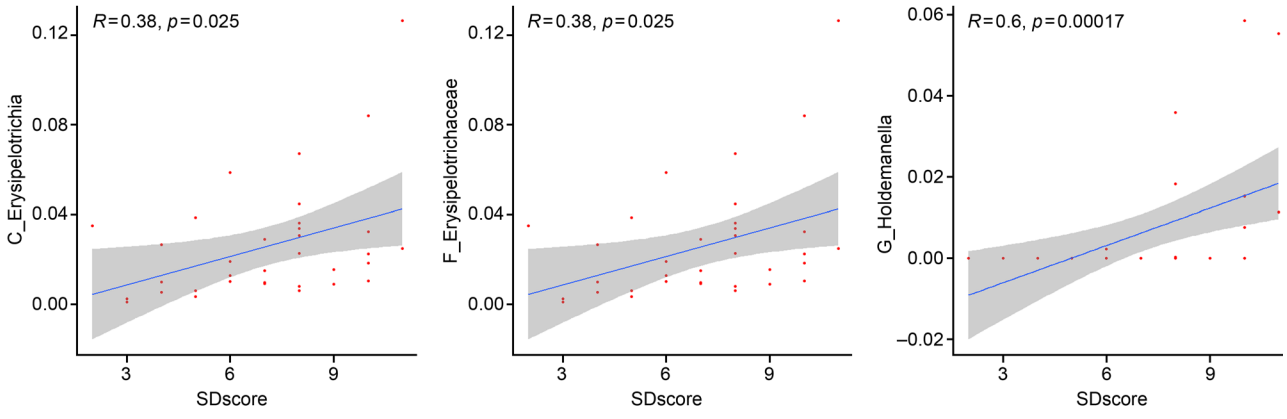

Fig. 5. Each point represents a sample and the 95% confidence intervals of the correlation coefficients are shown by grey areas. The relative abundances of genus *Holdemanella* in the samples were 0.

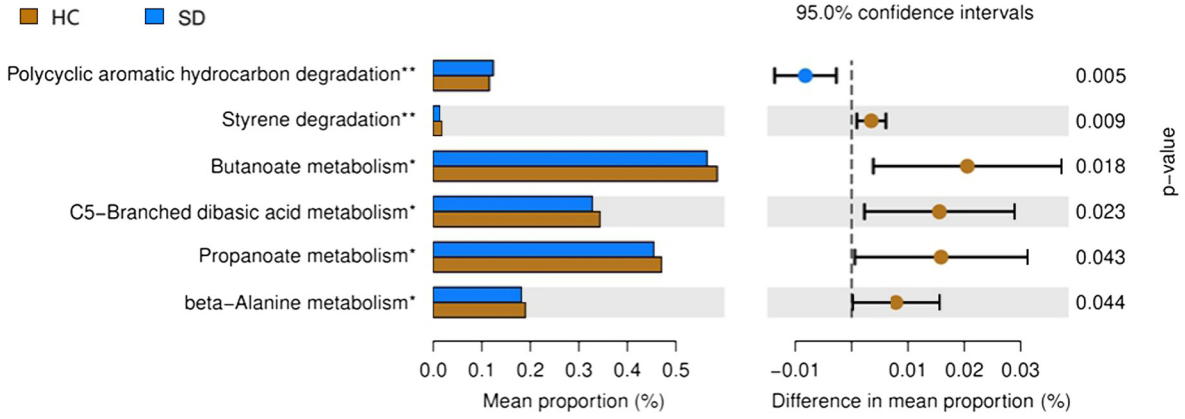

Fig. 6. Functional prediction analysis of the gut microbiota in the SD and HC groups. Each color represents one group. The bar graph represents the pathways with a significant difference in relative abundance between the two groups. The figure on the right shows 95.0% confidence intervals and *p*-value.

Discussion

Given that previous studies used mice models to verify the association between sleep disturbance and the gut microbiota and their interactions, we carried out the current study to determine whether poor sleep quality is associated with the gut microbiota in humans and, if so, whether more biomarkers can be determined for further study in the area. In the current study, a better balance of baseline characteristics between the SD and HC groups was reached after the screening of participants.

Although previous studies have suggested significant differences in the alpha diversity of gut microbiota between the sleep disorders and healthy populations, we failed to find any statistically significant differences in several parameters used to assess the alpha diversity of the gut microbiota. Moreover, the current results are consistent with those of Zhang et al. (2017), who showed that short-term sleep restriction did not significantly alter the OTU abundance, β diversity, and population shift of the gut microbiota in humans. In addition, the latest evidence has indicated that short-term sleep restriction and circadian misalignment do not appear to impair the stability of the alpha diversity of the human microbiome (Withrow et al. 2021). On the other hand, some researchers studied these relationships from a new perspective. They observed the changes in gut microbiota following short-term (two weeks) sleep extension in patients with chronic sleep deprivation, but no significant changes were found in this study (Reutrakul et al. 2020). Notably, the participants self-reported their current sleep conditions in our study, which may lead to the overestimated duration of SD in short-term partial subjects. However, recent results have shown a significant decrease in the abundance and alpha diversity of the gut microbiota in sleep disorders compared to healthy individuals (Liu et al. 2019). When there was disagreement between our results and some findings in previous studies, it is important to note the limitations of the present study and why some results should be interpreted with caution.

However, our study indeed found subtle significant differences in the structure of the gut microbiota in people with poor sleep quality compared to the sleep normal humans and finally identified eight taxa (q-value < 0.05), according to the result of Metastats analysis, which may be used to discriminate these two groups. Finding that sleep efficiency was positively correlated with the abundance of phylum Tenericutes has been mentioned in numerous previous studies (Benedict et al 2016; Reutrakul et al 2020; Withrow et al. 2020). Notably, phylum Tenericutes and class Mollicutes have been demonstrated to be resilient in response to acute sleep-wake cycle shifts (Liu et al. 2020). We also found that the relative abundance of the phylum Tenericutes (significantly, q-value < 0.05) and class Mollicutes (differences approaching significance, q-value = 0.069) in subjects with poor sleep quality were lower than in the healthy individuals. However, it remains to be determined whether this phenomenon represents that the taxa have the buffering capacity to some extent to external factors that may impair the host's sleep. In addition, we also observed a higher abundance of the family *Erysipelotrichaceae* (phylum Firmicutes) in participants with poor sleep quality (no significant difference after correction), which is consistent with the previous results (Benedict et al 2016). However, the latest evidence provided by Lin et al. (2021) indicated that *Lactobacillus fermentum* PS150 (PS150) could improve sleep conditions in the mice model and also induces gut microbiota remodeling, including an increased abundance of class Erysipelotrichia. Our findings do not confirm these results. One possible explanation for these discrepancies is species differences between humans and mice.

The PICRUSt prediction of the gut bacterial functions showed that several metabolic pathways differed significantly from healthy controls compared with individuals with poor sleep quality. However, some of them are challenging to interpret by current and previous evidence, and their clinical significance may be questionable, for instance, polycyclic aromatic hydrocarbon degradation. However, the predicted functional compositions of butanoate metabolism, C5-branched dibasic acid metabolism, and propanoate metabolism pathways were significantly down-regulated in the SD group. It is worth mentioning that sleep fragmentation could lead to a decreased abundance of putative butyrate-producing bacteria and further affect the butanoate metabolism (Maki et al. 2020). As the members of specific short-chain fatty acids (SCFAs) (Magzal et al. 2021), butanoate has also been demonstrated to play a role in inducing sleep in mice models (Szentirmai et al. 2019). Additionally, butanoate has some functions similar to SCFAs, such as energy metabolism and inflammation of the host (Hamer et al. 2008), and pieces of previous evidence have suggested that changes in microbiota composition induced by the circadian rhythm misalignment might have implications for inflammatory diseases (Voigt et al. 2014; Parkar et al. 2019; Mashaqi and Gozal 2020). C5-Branched dibasic acid metabolism and propanoate metabolism in patients with obstructive sleep apnea-hypopnea syndrome has also been found with significant differences compared to healthy humans (Li et al. 2017; Ko et al. 2019). Correspondingly, the increased concentration of propanoate in human infant fecal samples was associated with the prolongation of sleep duration (Heath et al. 2020). Significant differences in the gut microbiota composition and function were observed in our study, which further demonstrated the relationship between sleep problems and the gut microbiota in humans, but the causality remains to be fully explored.

This study's findings must be considered along with limitations. Initially, the observational characteristic of the cross-sectional study cannot provide enough evidence for supporting the potential causal links between poor sleep quality and changes in the gut microbiota. Secondly, further study on larger samples is also needed to identify the other key bacteria, reported in a previous study but failed to be found in this study. Moreover, in studies with small sample size, the change of statistical methods may need to be considered. As Weiss et al. (2017) mentioned, DESeq2 could increase sensitivity on smaller datasets (< 20 samples per group). In addition, the lack of multiple objective measures of sleep (e.g., electroencephalogram, polysomnography) could reduce the accuracy and timeliness of the data and induce recall bias due to the self-reported approach.

On the other hand, the gut microbiota composition may be influenced by diet. So, in future research, the dietary survey of the participants should be added and included as a control variable for reducing the influence on gut microbiota. Finally, as a preliminary exploration, we failed to find robust evidence from the current research to establish the association between the bacteria that changed and the metabolic pathways identified. Therefore, future studies are warranted to further examine the causal relationship between sleep and gut microbiota by considering more other factors.

Conclusions

In conclusion, our study demonstrated an association between the composition of the gut microbiome and poor sleep quality. Moreover, we identified several specific phyla and taxa (e.g., Elusimicrobia and Tenericutes) that may be used to distinguish sleep-disordered patients from healthy individuals. Our results also revealed the difference in functional pathways, such as butanoate metabolism and propanoate metabolism, which raise the possibility that the gut microbiota could be a potential contributor to diagnosing and treating sleep problems in the future.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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