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Correlation between symptoms and cognitive function changes in patients with primary insomnia and pathways in gut microbiota

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ABSTRACT

Background: Primary insomnia (PI) refers to syndromes of difficulty falling asleep, poor sleep quality, early awakening, and difficulty falling asleep after waking up. Although there have been numerous studies, the specific etiology and pathogenesis of PI are still misunderstanding. In recent years, the gut microbiota has been proved to be involved in the metabolism of many mental disorders. But the specific mechanisms of its involvement in PI have not been fully elucidated. This study aims to explore the relationship between the gut microbiota and the symptoms, cognitive function changes in PI.

Methods: In this study, the gut microbiota of PI patients and healthy controls was profiled by performing stool 16s rRNA gene sequencing. The co-occurrence network was constructed by using Weight Gene Co-expression Network Analysis (WGCNA) algorithm. The correlation between gut microbiota associated pathways and traits in PI were predicted.

Results: WGCNA results demonstrated several Operational Taxonomic Units (OTU) modules are correlated to symptoms. By using PICRUSt2 software, we predicted the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of microbiota in modules. For instance, sleep efficiency may be correlated with the presence of Insulin signaling pathway, Flavonoid biosynthesis, Ascorbate and aldarate metabolism, Nitrotoluene degradation, Biotin metabolism, RNA polymerase and Chlorocyclohexane and chlorobenzene degradation. Total sleep time may be correlated with the presence of Tyrosine metabolism, Propanoate metabolism, Carbon fixation pathways in prokaryotes, Carotenoid biosynthesis, Systemic lupus erythematosus, Nitrotoluene degradation and Biosynthesis of unsaturated fatty acids. The severity of insomnia may be correlated with Insulin signaling pathway, Flavonoid biosynthesis, Ascorbate and aldarate metabolism, Nitrotoluene degradation, Biotin metabolism and RNA polymerase. Change of name score in Montreal Cognitive Assessment (MoCA) may be correlated with Tyrosine metabolism, Propanoate metabolism, Carbon fixation pathways in prokaryotes, Carotenoid biosynthesis, Systemic lupus erythematosus, Nitrotoluene degradation, Biosynthesis of unsaturated fatty acids, Apoptosis, Steroid hormone biosynthesis, Geraniol degradation, Protein digestion and absorption and Bisphenol degradation in Gut Microbiota (GM).

Conclusion: This study revealed the potential relationships between gut microbiota and PI. By using pathway prediction and enrichment analysis, we concluded many metabolic pathways may associated with some important traits of insomnia patients, including sleep efficiency, severe insomnia, total sleep time and change of name score in MoCA. The metabolic pathways include Insulin signaling pathway, Flavonoid biosynthesis, Ascorbate and aldarate metabolism, Nitrotoluene degradation, Biotin metabolism, RNA polymerase and Chlorocyclohexane, chlorobenzene degradation, Tyrosine metabolism, Propanoate metabolism, Carbon fixation pathways in prokaryotes, Carotenoid biosynthesis, Systemic lupus erythematosus, Biosynthesis of unsaturated

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fatty acids, Apoptosis, Steroid hormone biosynthesis, Geraniol degradation, Protein digestion and absorption and Bisphenol degradation.
Our study demonstrated that PI patients demonstrate significant changes in gut microbiota, which will help delineate the relationship between gut microbiota and syndromes of PI.

1. Introduction

Primary insomnia (PI) is one of the most common sleep problems characterized by difficulty initiating sleep or maintaining sleep or early awakening for at least 1 month that can't be explained by the use of medications, physical disorders, pain or other mental illnesses [1]. It is a group of chronic, recurrent disorders that cause different degrees of impairment in mental activity such as behavioral, emotional and cognitive activities, presenting a continuous trajectory of disease development. Epidemiological studies showed that approximately 35 %–50 % of adults suffer from insomnia symptoms and the prevalence of insomnia disorder ranges from 10% to 30 % of the population world [2,3]. Additionally, primary insomnia impairs daytime function such as fatigue, mood disturbance, diminished cognitive performance (memory, attention, concentration) [4,5].

Insomnia is often treated by pharmacological therapies and the non-pharmacological treatment (i.e. cognitive behavioral therapy), but pharmacological treatment often causes adverse effects or drug dependence, while cognitive behavioral therapy is limited due to number of qualified therapists and high costs [6,7]. Hence, there is a need to study the underlying mechanism of insomnia to develop more treatments. There have been many studies on the physiological and pathological mechanism of PI, but few people have explored whether changes in the microbial environment in the gut will be an important cause of primary insomnia.

GM is now known to be closely linked to human brain activity. Previous experimental studies have confirmed that Microbiota-Brain-Gut Axis (MBGA) is correlated with degenerative changes in the brain activities [8]. The signal transmission mechanism in MBGA is very complex, involving two-way communication. The gut microbiota can communicate with the brain, and the brain can also transmit information to the gut microbiome [9]. Therefore, the symptoms and cognitive changes in PI patients are likely to be related to changes in GM. Li Y et al. found that the GM can interact with the human circadian rhythm, and the characteristics of the GM and metabolism are related to the host's sleep and circadian rhythm [10]. Although previous studies have revealed that GM has a strong relationship with cognitive function changes [11,12], they have not been able to explain the specific mechanism of MBGA. Which flora and pathways in GM affect brain activities is still a problem. Based on previous research [13,14], this experiment aims to explore whether symptoms and cognitive changes in patients with PI are related to the presence of specific pathways in GM.

GM is key to many aspects of human diseases. Recent evidence has linked GM to many kinds of neurobehavioural traits, which suggested that GM may be a promising biomarker of mental disorders. Chang L et al. found that alterations in the GM, microbiome-derived short-chain fatty acids, D-amino acids, and metabolites play a key role in the pathophysiology of depression through the MBGA [15]. Simpson C et al. believe that the increase in the relative abundance of pro-inflammatory species and the decrease in the relative abundance of short-chain fatty acid producing-species are likely to be one of the important causes of anxiety and depression [16]. Therefore, it is worthwhile to explore the relationship between GM and PI.

16s rRNA sequencing has been widely used in the studies of the correlation between GM and neurological diseases. Palkova L et al. used 16s rRNA primer sets to evaluate the gut microbiota of children with autism spectrum disorder (ASD) [17]. Xu Y et al. used 16s rRNA microbial data to explore the composition and structure of fecal microbiota in patients with ASD, and found that there were significant differences in the composition and structure of fecal microbiota in ASD patients compared with healthy controls [18]. Fujishiro S et al. explored the characteristics of the GM of children born preterm with ASD and typically developing (TD) children born preterm through 16s rRNA sequencing, and found that the GM of children born preterm differed due to ASD or TD [19]. Through 16s rRNA gene and metagenomic sequencing analysis, Peng W et al. found that the perturbations of GM components and functional metagenomes may be related to Alzheimer's Disease (AD) [20]. Yu M et al. found that 16s rRNA gene sequencing and LC-MS based metabolomics can be applied to evaluate the pathogenesis of depression [21]. Hong J et al. found through 16s rRNA sequencing analysis that acupuncture treatment can improve sleep disorders in mice by affecting GM [22]. Thus, we hypothesis that GM may be a valuable factor of PI. We collected the stool samples of PI patients and healthy controls and employed 16s rRNA sequencing to comprehensively investigate the correlation between GM and PI according to multiple bioinformatics methods.

2. Materials and methods

2.1. Subjects

The sample size of this study is 67, including 38 in the insomnia group and 29 in the control group. From August 2019 to June 2021, 38 primary insomnia patients and 29 matched healthy subjects without insomnia were recruited from imaging department of Guangdong Second People's Hospital. Demographic information and medical history were collected from the participates, and the Pittsburgh Sleep Quality Index (PSQI), the Insomnia Severity Index (ISI), the Self-Rating Anxiety Scale (SAS), the Self-Rating Depression Scale (SDS), Trail Making Test, Rey Auditory Verbal Learning Test (RAVLT), Digit Symbol Test (DST), Digit Span Test and Montreal Cognitive Assessment (MoCA) were measured. Stool samples from participants were then collected and intestinal flora was examined. Then, the two cohorts (38/29) matched with the three kinds of data of clinical symptoms-behavioral scale-GM were analyzed in depth, in order to clearly and systematically indicate the possible intestinal biological mechanism of abnormal brain changes caused by insomnia.

Potential PI candidates for enrolment in the study satisfied the following criteria.

Inclusion criteria.

(1) All patients met the diagnostic criteria for primary insomnia according to Diagnostic and Statistical Manual of Mental Disorders, version 4 (DMS-IV) [23].
(2) Patients between the ages of 21–71, of either gender.
(3) Patients with a PSQI score of > 5.
(4) Patients with an ISI score of > 7.
(5) Right-handedness.

Exclusion criteria.

(1) Patients with mixed affective disorder, schizophrenia, and other severe mental disorders.
(2) Patients with other sleep disorders such as narcolepsy, circadian rhythm disorder, sleep walking, restless legs/periodic limb movement disorder or sleep-disordered breathing.
(3) Patients with treatment for insomnia involving any hypnotic or sedating medications within a 1-month period.

Table 1
Demographics and clinical data comparisons.

Characteristics	PI	HC	Statistics	*P*-value
case	38	29	N/A	N/A
Gender (M/F)	7/31	7/22	$\chi2 = 0.325$[a]	0.568
Age (years old)	46.4 ± 15.4	47.3 ± 10.5	T = −0.285[b]	0.776
Education (years)	12.8 ± 3.1	11.2 ± 5.3	T = 1.544[b]	0.127
Age of onset (years old)	42.3 ± 15.3	N/A	N/A	N/A
Illness duration (months)	61.6 ± 49.7	N/A	N/A	N/A
Sleep-onset latency	55.7 ± 49.5	N/A	N/A	N/A
Sleep efficiency	0.7 ± 0.1	N/A	N/A	N/A
Total sleep time	5.1 ± 1.0	N/A	N/A	N/A
PSQI scores	12.9 ± 2.9	N/A	N/A	N/A
ISI scores	16.5 ± 4.6	N/A	N/A	N/A
SAS scores	49.1 ± 11.0	N/A	N/A	N/A
SDS scores	52.8 ± 13.0	N/A	N/A	N/A

Notes: Means and standard deviations (SD) are listed in the table.
Abbreviations: PI, Primary insomnia; HC, healthy control; M, male; F, female; PSQI: The Pittsburgh Sleep Quality Index; ISI: The Insomnia Severity Index; SAS: Self-Rating Anxiety Scale; SDS: Self-Rating Depression Scale.
N/A, not applicable.
[a] $\chi2$-test.
[b] Two-sample *t*-test.

Table 2
Neuropsychological tests and cognitive assessment data comparisons.

Characteristics	PI	HC	Statistics	*P*-value
Trail Making Test A scores	57.7 ± 24.4	62.2 ± 26.1	Z = −0.665[c]	0.506
Trail Making Test B scores	159.6 ± 82.8	160.1 ± 73.6	Z = −0.127[c]	0.899
RAVLT - immediate recall scores	39.1 ± 9.9	41.3 ± 10.9	Z = −0.825[c]	0.409
RAVLT - delayed recall scores	2.8 ± 1.6	3.2 ± 2.1	Z = −1.622[c]	0.105
Digit Symbol Test scores	43.7 ± 17.9	44.5 ± 17.3	Z = −0.386[c]	0.699
Digit Span Test scores	13.5 ± 2.3	13.6 ± 1.9	Z = −0.493[c]	0.622
MoCA - total scores	24.1 ± 3.2	25.2 ± 3.4	Z = −1.464[c]	0.143
MoCA - visuospatial/ executive scores	3.6 ± 1.3	3.9 ± 1.9	Z = −2.297[c]	0.022
MoCA – Naming scores	2.8 ± 0.5	2.8 ± 0.5	Z = −0.722[c]	0.470
MoCA – memory scores	3.5 ± 1.3	2.9 ± 1.5	Z = −1.657[c]	0.098
MoCA – attention scores	5.0 ± 1.2	5.6 ± 0.7	Z = −1.910[c]	0.056
MoCA – language scores	2.2 ± 0.7	2.1 ± 0.8	Z = −0.547[c]	0.585
MoCA – abstraction scores	1.6 ± 0.6	1.4 ± 0.9	Z = −0.733[c]	0.464
MoCA - delay recall scores	2.4 ± 1.4	3.3 ± 0.8	Z = −2.657[c]	0.008
MoCA – orientation scores	6.0 ± 0.0	6.0 ± 0.0	Z = 0.000[c]	1.000

Abbreviations: PI, Primary insomnia; HC, healthy control; RAVLT: Rey's Auditory Verbal Learning Test; MoCA: Montreal Cognitive Assessment.
[c] Mann-Whitney *U* test.

(4) Patients with a history of alcohol/drug abuse or dependence.
(5) Pregnant or lactating women.

2.2. 16s rRNA gene sequencing and bioinformatics analysis

All participants were collected stool samples after completing all questionnaires and screened to perform gut microbiota profiling by 16s rRNA gene sequencing. First, stool samples were collected and stored in a sterile container within a −80 °C refrigerator until use.

Total DNA was isolated from the stool samples (0.5 g) by using the FastDNA SPIN Kit for Feces (MP Biomedicals, United States) in a FastPrep Instrument (MP Biomedicals), according to the manufacturer's instructions. The quality and concentration of the DNA were tested using Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). PCR amplification of 16s rRNA gene sequences was performed using primer sets 338F (ACTCCTACGGGAGGCAGCA) and 806 R (GGACTACHVGGGTWTCTAAT) which were specific for V3–V4 regions. Final PCR products were purified. Purified samples were normalized to equal DNA concentration for library preparation. Library sequencing were performed by Illumina MiSeq platform (Illumina, San Diego, USA) in PE250 read lengths.

After quality control and filter, the clean reads were used to perform the next bioinformatics analysis. Raw reads were filtered to remove low-quality and ambiguous bases, and then paired-end reads were added to tags by the Fast Length Adjustment of Short reads program (FLASH, v1.2.11) [24] to get the tags. Tags containing primers were removed using CUTADAPT [25]. Tags containing chimeras were removed using VSEARCH (v2.3.4) [26] with UCHIME method by aligned to gold database (v20110519) Chimeras database. Then, tags were clustered into OTUs with a cutoff value of 97 % using VSEARCH (v2.3.4). OTU representative sequence were aligned to Silva (V128) database using RDP Classifer (V2.2) [27] software for species annotation. Alpha diversity were estimated by mothur (v1.39.5) [28], 5 index were calculated, including Chao, Observed species, ACE, Shannon, and Simpson. Beta diversity was estimated by QIIME (v1.8.0) [29] at the OTU level. The other figures were plotted with R package (v3.0.3). LDA score was computed for differentially abundant between the two groups by LEfSe (LDA Effect Size) (v1.0) [30]. $P < 0.05$ and log10 [LDA]$\geq$2.0 (or ≤ -2.0) was considered significant. KO, EC, PFAM, TIGRFAM, COG, MetaCyc, KEGG functions were predicted using the PICRUSt (v2.3.0-b) software [31]. The methods to analyze the OTUs included OTU Venn Graph, Principal Component Analysis, OTU Rank Curve, and species accumulation curves. The analyses of relative abundance included bacterial profiling, construction of a heatmap, and genus specific phylogenetic tree.

In order to detect relationships between gut microbiome and their potential combined effect on phenotypes of PIs, WGCNA was performed to construct a network of cooccurrence OTUs based on all normalized OTU count data, the network was clustered into several modules, the correlationship between modules and phenotypes of PI was calculated.

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database of genome deciphering. It is a comprehensive database resource for biological interpretation of genome sequences and other high-throughput data. The link between the molecular function of genes and proteins is stored in the KEGG database [32]. To predict the enriched KEGG pathways of modules, functional analysis was performed using PICRUSt2. The Wilcoxon rank-sum test was used to compare the differences between pathways.

3. Results

3.1. Assessment of sleep quality, mental status and cognitive ability

Prior to sample collection, each participant was asked in detail about their personal data. PI patients were also asked in detail about insomnia-related medical history. All participants were asked to undergo a series of standardized neuropsychological assessments. The observations were divided into demographic data, sleep-related data, and neuropsychological assessments. Demographic data included gender, age, move, height, weight and education years. Sleep-related data included insomnia duration, age of insomnia, sleep onset latency and total sleep time. Neuropsychological assessments included PSQI, ISI, Mild insomnia, Moderate insomnia, Severe insomnia, SAS, SDS, HAMA, Trail Making Test, RAVLT, DST, Digit Span Test and MoCA. The demographic data and the sleep-related data comparisons for the included subjects are

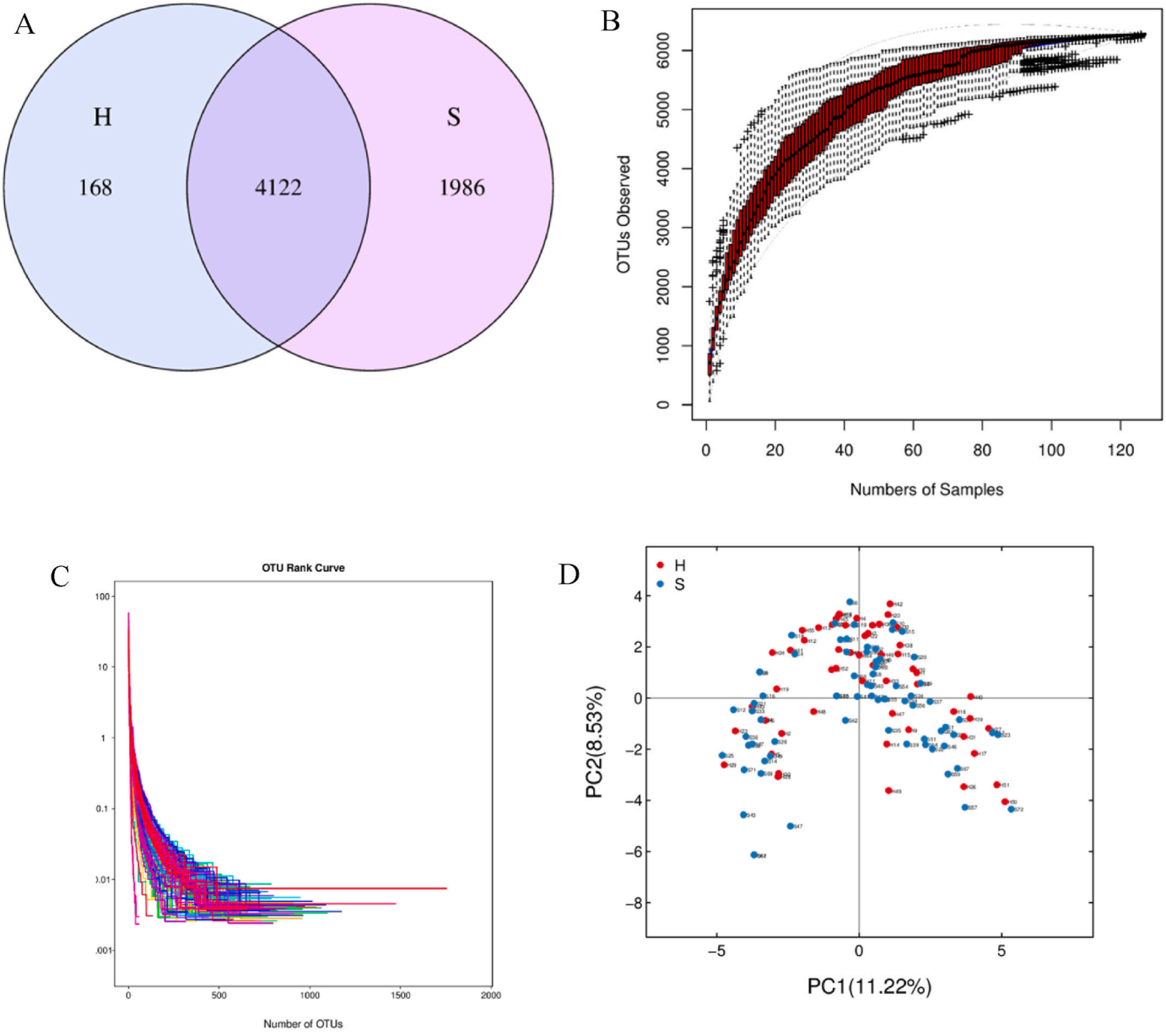

Fig. 1. A–D The Gut Microbial Structure among PI and HCs. A, Veen plot of OTUs between PI and HC cohort; B, Species accumulation curves of all samples; C, The relative abundance rank curves of OTUs in each sample; D, 2D PCA plot of all samples.

Table 3
The TOP10 phylum relative abundance in two cohorts.

	Cohorts	
	HC	PI
Firmicutes	68.24455	52.51206
Bacteroidetes	13.28049	33.57886
Actinobacteria	13.192	7.285218
Proteobacteria	4.453263	4.906191
Fusobacteria	0.386309	1.324262
Verrucomicrobia	0.41137	0.231078
Synergistetes	0.005674	0.10556
Unclassified	0.013645	0.025059
Tenericutes	0.008376	0.016854
Lentisphaerae	0.000338	0.009425

shown in Table 1. The neuropsychological tests and cognitive assessment data comparisons for the included subjects are shown in Table 2.

3.2. Comparison of gut microbiota between PI and HC

In order to investigate the difference of gut microbiota between PI and HC, 16s rRNA sequencing was performed with the total DNA extracted from the stool samples.

After quality control and filter, total clean reads pair number of 67 samples was 9439948. Species diversity analysis of each sample was performed with Alpha diversity analysis (including observed species, Chao, Ace, Shannon and Simpson) to estimate fecal microbial species richness and species diversity. The average OTUs counts of PI samples and HC samples were 678 and 650, respectively. The common OTUs counts of two cohorts was 4122 (Fig. 1A, all figures in this article were made using R software). The relative abundance of each OTU was calculated. The rank curve showed the distribution of OTUs relative abundance in each sample (Fig. 1C). Principal Component Analysis

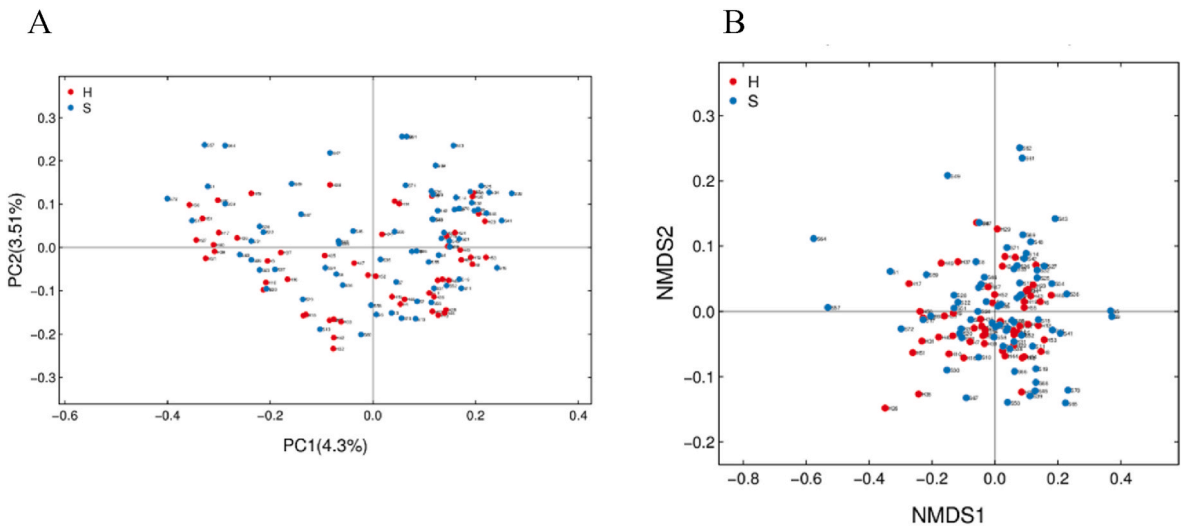

Fig. 2. A–B PCoA and NMSD analysis. A, 2D PcoA plot of all samples; B, NMDS plot of all samples.

(PCA) was performed at OTU level, two axes explained 11.22 % and 8.53 % of the total variation in PI and HC samples (Fig. 1D). Species accumulation curves showed the species richness of all samples (Fig. 1B).

By mapping the OTUs to database Silva (Version: 128), OTUs were annotated (Fig. 4). The most abundant species in two cohort at phylum level are exhibited in Table 3 and supplement materials. Alpha diversity analysis were performed to access the gut microbiota diversity of each sample. The sobs index, chao index, ace index, shannon index and simpson index were calculated and exhibited in supplement materials. Meanwhile, Principal coordinates analysis (PCoA) and Nonmetric Multidimensional Scaling (NMSD) analysis were performed to indicate the species diversity between samples and cohorts, indicating the differential component of gut microbiota in each sample and cohort (Fig. 2A and B) (see Fig. 5) (see Fig. 3).

Next, the differential analysis was performed by using Anosim test, the test statistic of unweighted unifrac and bray curtis method were above of 0 (0.024262171329728844 for unweighted unifrac and 0.038712164955669079 for bray curtis), but only the P value of bray curtis method were Less than 0 (P value = 0.027). The linear discriminant analysis effect size (LEfSe) was chosen to illustrated the relative abundances at the phylum, class, order, family, genus and species.

3.3. Weight gene Co-expression Network Analysis (WGCNA)

Based on the OTU abundance results, we chose WGCNA method to build a correlation matrix containing all pairwise correlations between all OTUs across all samples. WGCNA has been applied to complex microbial communities in several reports. WGCNA algorithm assumes that the probe network follows a scaleless distribution, and defines the probe co-expression correlation matrix and the neighbor function formed by the gene network, and then calculates the difference coefficients of different nodes, and constructs a hierarchical clustering dendrogram, according to which different branches of the cluster dendrogram represent different gene modules, and the co-expression degree of genes in the module is high, while the co-expression degree of genes belonging to different modules is low. Finally, the module explores the association relationship between specific phenotypes or diseases, and finally achieves the purpose of identifying target genes and gene networks for disease treatment (see Fig. 6).

First, the correlation values of OTUs were calculated to define the gene co-expression network. Then, the OTUs abundance similarity matrix was converted into and an adjacency matrix, based on the criterion of approximate scale-free topology, to define the strength between connected OTUs. The soft thresholding power value was selected as $\beta = 11$ (scale-free $R^2 = 0.80$) to ensure a scale-free network (Fig. 7).

Modules of co-occurrence OTUs were then determined by using the dynamic tree cut procedure. After OTUs module determination, the EigenOTU of each module were calculated, modules were clustered to merge the modules with a similarity higher than 80 % into new modules. With this procedure, 13 different modules were identified (Fig. 7). The size of modules ranges from a minimum of 52 OTUs of the Tan module to the 153 OTUs of the Turquoise module. But 1352 OTUs were not classified in any of the colored modules and then included in the Grey one. The TOM plot of the OTUs network was generated with the corresponding hierarchical clustering dendrogram and the modules.

The correlationship between traits and the module EigenOtus (MEs) were performed for each module and the results were summarized in a heatmap. A few statistically significant correlations were found with respect to the PI traits. It can be concluded from the ME-traits correlation results that sleep efficiency is strongly correlated with MEyellow, MEtan and MEblack, total sleep time with MEblue, Severe insomnia with MEblack, HAMA with MEgreenyellow, MoCA Name with MEblue and Mepink (Fig. 8).

3.4. KEGG enrichment analyses and Wilcoxon test

By WGCNA analysis, we have obtained a grouping of modules with highly correlated traits. KEGG enrichment analyses based on the results of this grouping allows analysis of the enrichment of relevant pathways in stool samples. This makes it possible to find PI-related pathways in GM.

In this study, we chose yellow module, tan module and black module for sleep efficiency, tan module, blue module for sleep time, black module for severe insomnia, greenyellow module for HAMA, blue module and pink module for MoCA name to perform the KEGG pathways enrichment. The *P*-value cut-off of significant difference by Wilcoxon test were less than 0.03 (Table 4).

4. Discussion

The objective of this study was to explore the relationship between the symptoms and cognitive function changes in PI patients and the potential pathways present in the GM. For this, we conducted a series of general information interrogations of all subjects, as well as a series of standardized scale tests, and finally collected their stool samples.

Many reports indicate that the gut microbiota is associated with various neurobehavioral characteristics. In this study, we analyzed the

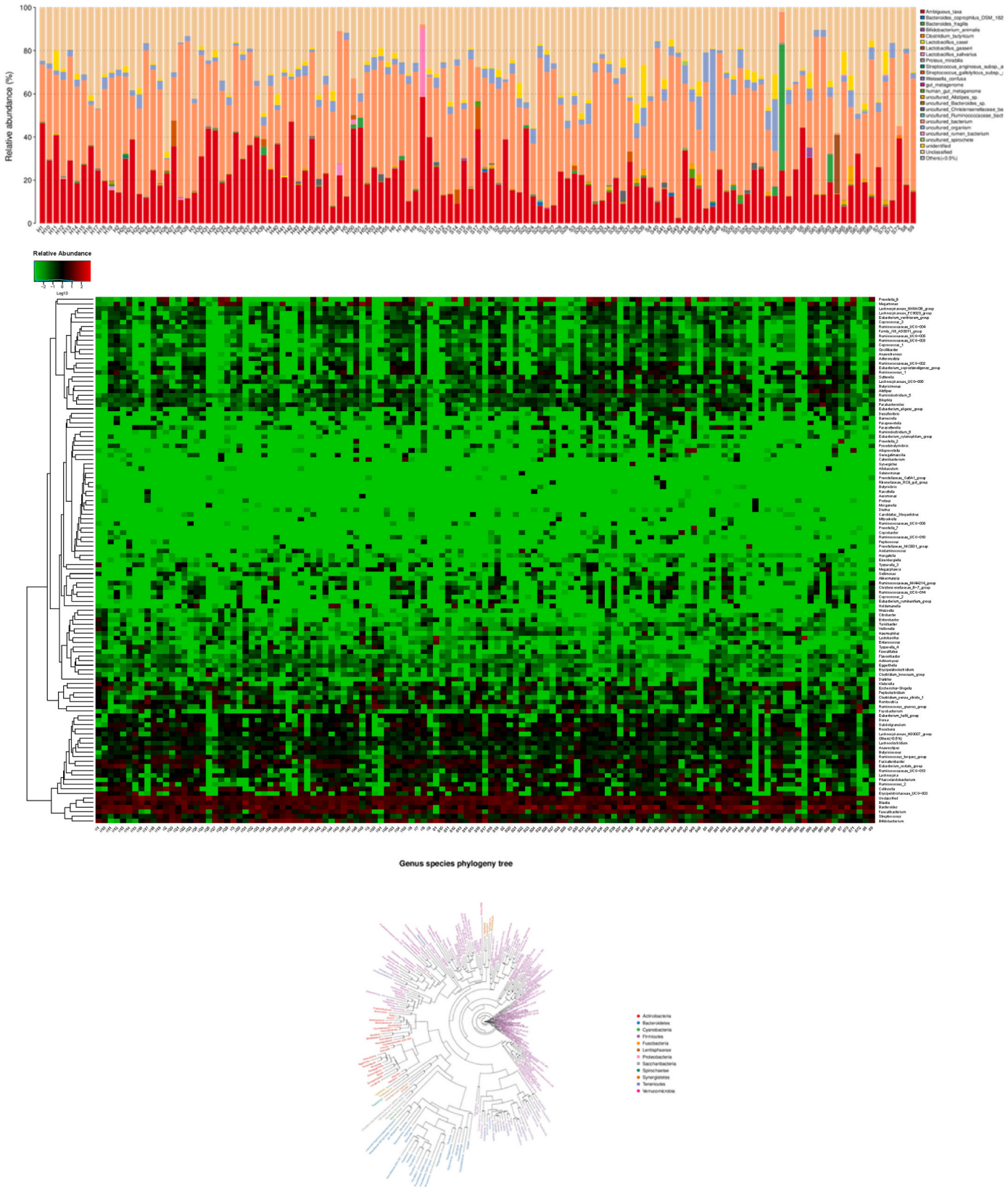

Fig. 3. The annotation of OTUs in all samples. A, Relative abundance of the most abundant OTUs at the species level in all samples. B, Heatmap of the most abundant OTUs at the genus level in all samples. C, The gut microbiota cladogram in genus level of all samples.

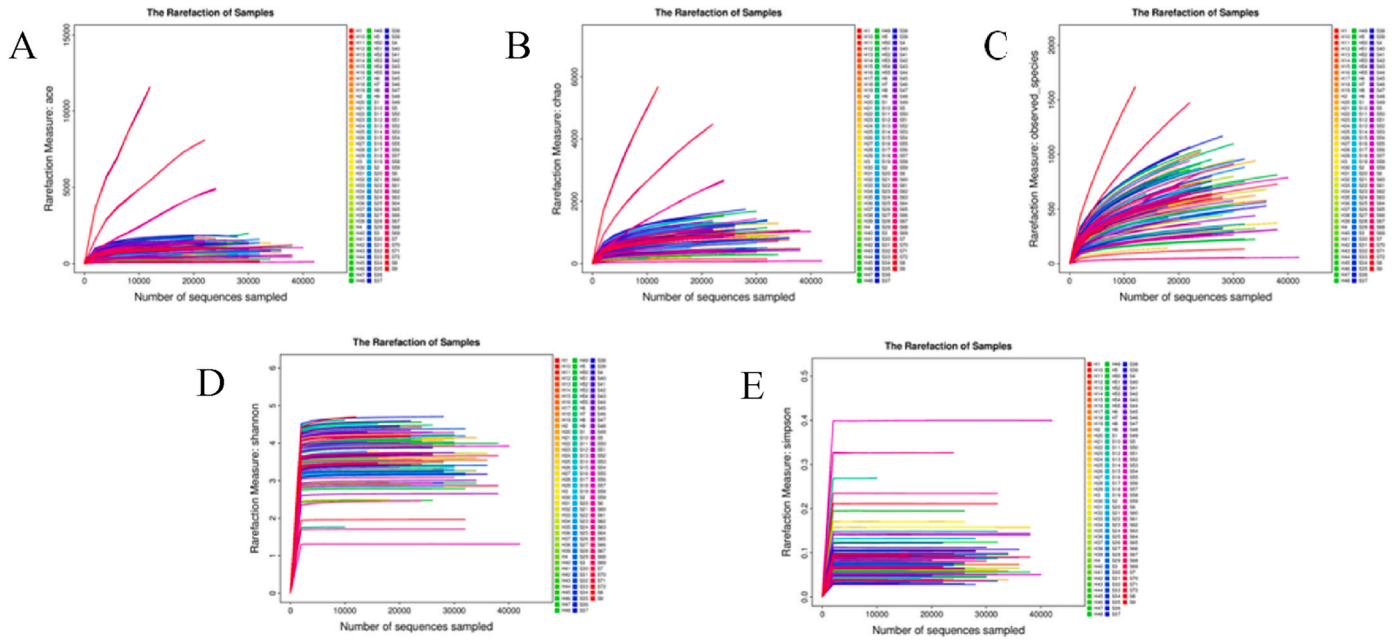

Fig. 4. Alpha Diversity analysis results. A, the Alpha index Rarefaction Curve of all samples. B, the chao index Rarefaction Curve of all samples. C, the observed species Rarefaction Curve of all samples. D, the shannon index Rarefaction Curve of all samples. E, the simpson index Rarefaction Curve of all samples.

gut microbiota of 38 insomnia patients (PIs) and 29 healthy controls (HCs) by 16s rRNA sequencing. The average OTUs counts of PI samples and HC samples was 678 and 650, respectively. The common OTUs counts of two cohorts was 4122. Two cohorts (38/29) matched with the three kinds of data of clinical symptoms-behavioral scale-GM were analyzed in depth. As shown by the results of LEfSe, PCA, PCoA and NMDS analysis, we observed that the gut microbiota underwent evident structural changes including community composition and relative abundance.

In this study, we found that at the phylum level, the relative abundance of Firmicutes and Actinobacteria in the GM of PIs decreased significantly compared with HCs, while the relative abundance of Bacteroidetes increased significantly (Table 3). This may suggest that changes in the abundance of species in GM may affect human neurophysiological function. Huang Y et al. found that the relative abundance of Firmicutes in the gut microbiota of people with depression was significantly reduced. A decrease in the relative abundance of Firmicutes may lead to a deficiency of short-chain fatty acids, which may be the physiological basis for low levels of depression [33]. Knudsen JK et al. believe that the relative abundance of Eggerthella, Atopobium and Bifidobacterium in patients with major depressive disorder (MDD) increases and the relative abundance of Faecalibacterium decreases compared to healthy controls [34]. Lee HJ et al. found that probiotic NVP-1704, a mixture of Lactobacillus reuteri NK33 and Bifidobacterium adolescentis NK98, has significant efficacy in improving stress, depression, anxiety, and sleep quality [35]. Nishida K et al. found that long-term use of tablets containing Lactobacillus gasseri CP2305 can improve mental state, sleep quality and GM in healthy adults under stressful conditions [36]. Gamma-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system (CNS) [37]. Its metabolic disorder is related to anxiety and depression [38]. Duranti S et al. found that Bifidobacterium adolescentis taxon may be a major producer of GABA in the GM [39]. Lactobacillus and Bifidobacterium belong to Firmicutes and Actinobacteria, respectively. The conclusions of these studies are consistent with ours, which may provide a new therapeutic strategy for clinical management of neurophysiological diseases such as anxiety, depression, and insomnia. That is, increasing the relative abundance of Firmicutes and Actinomycetes in GM through probiotics may help to treat primary insomnia.

Probiotics are lactic acid-producing bacteria that typically include Bifidobacterium and Lactobacillus species, which are beneficial to human health [40]. McKean J et al. showed through a meta-analysis that probiotic supplementation in healthy people may have a positive effect on their psychological symptoms of depression, anxiety and stress [41]. The specific mechanism of probiotics affecting human neurophysiological function through MGBA is still unclear. It is thought that the MGBA mainly contains three main pathways: neural pathways, neuroendocrine pathways, and immune pathways [42]. But exactly which signal pathways are involved in MGBA needs to be studied in depth.

Due to the comprehensive biological significance of WGCNA [43,44], it is suitable to be applied to explore the relationship between gut microbiota OTUs, modules and clinical traits. Functionally related OTUs in modules that are highly correlated with PI traits can be considered as biomarkers for clinical testing and treatment.

After co-expression network construction, 13 modules were identified. We calculated the correlation coefficient between modules and PI traits and did the KEGG pathway enrichment analysis by using PICRUSt2 and compare the differences between pathways in Wilcoxon rank-sum test. We screened out 8 sets of module-trait combinations with high correlation. They are sleep efficiency with modules black, tan and yellow, total sleep time with module blue, severe insomnia with module black, HAMA with module yellow, as well as MoCA name with module blue and pink.

After Wilcoxon's test, pathways with strong correlation with traits were selected ($P < 0.03$). Traits associated with patients with PI may be related to the pathways present in GM.

The relationship between the relevant traits of insomnia patients and the presence of pathways in GM is detailed in Table 4 ($P < 0.03$). The pathways listed in the table may be involved in the regulation of neurophysiological functions by GM through MGBA.

In this study, we found that sleep efficiency may be correlated with the insulin signaling pathways present in GM. In previous studies, Piromelatine is a novel investigative multimodal sleep medicine used to treat patients with primary and comorbid insomnia. Piromelatine has been shown to improve insulin sensitivity in rats [45]. Soto M et al. also found that changes in the gut microbiota affect the level of insulin signaling pathways in the brain, which in turn affects neural behavior [46].

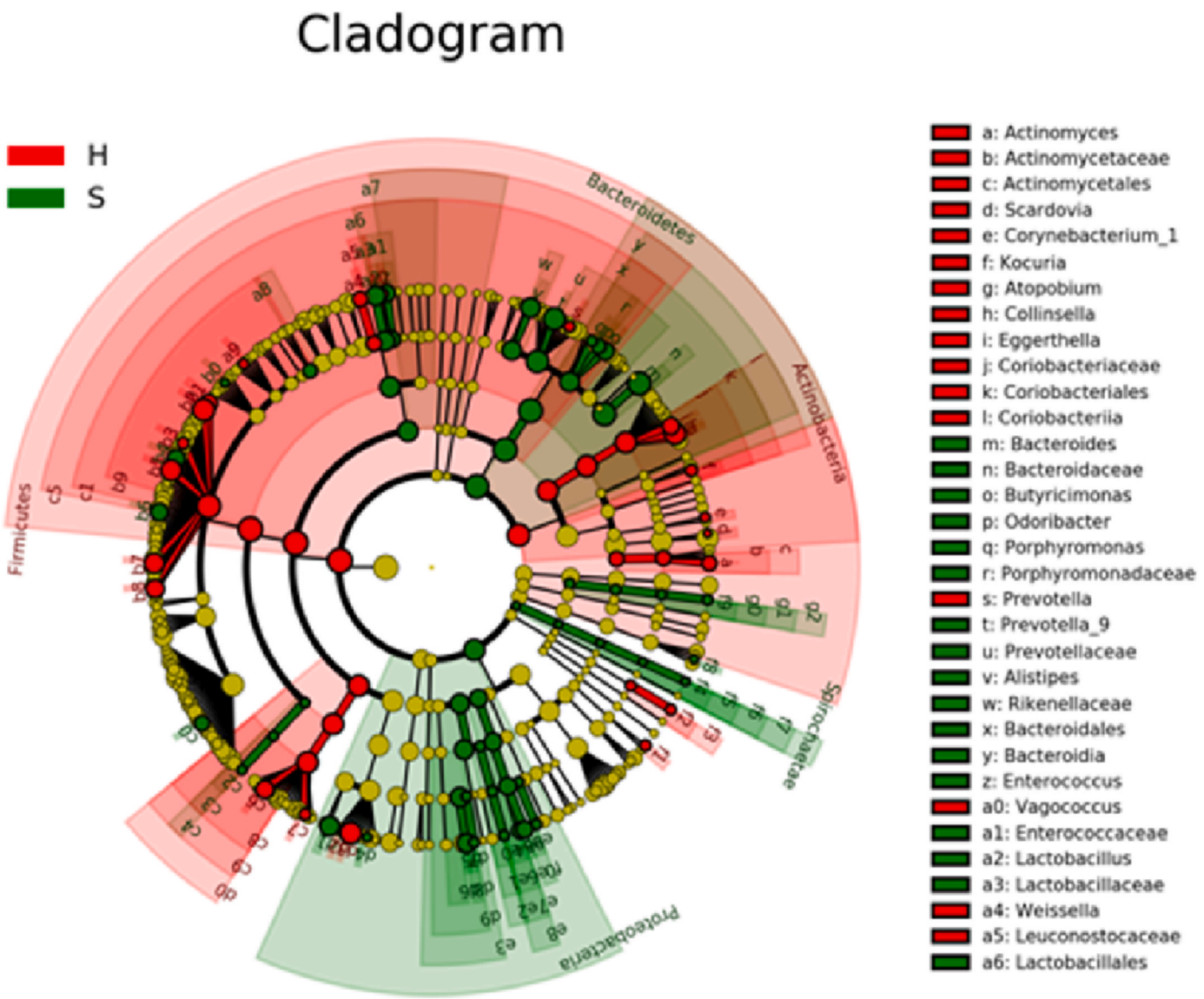

Fig. 5. Linear discriminant analysis effect size. The LEfSe Cladogram plots demonstrate the biological structure of the gut microbiota. Gut microbiota marked with small circles highlight significant differences of relative abundance between the two cohorts.

Flavonoids are bioactive substances that are beneficial to human health. Zhao K et al. showed that flavonoids are metabolized by GM to form a variety of different derivatives, which can have beneficial effects on the intestine and can improve depressive symptoms through MGBA [47]. Ashwagandha (ASH) is a traditional botanical medicine of Indian medicine that can be used to treat neurasthenia, memory-related disorders, insomnia, and improve learning and memory ability. Studies have shown that the metabolite profiling of ASH reveals more than 45 recognized metabolites, including flavonoids [48]. Flavonoids can also be used to treat cognitive decline. Dietary flavonoid supplementation can improve cognitive impairment through GM [49], which provides a new strategy for the clinical treatment of cognitive dysfunction.

Biotin is one of the B vitamins. Its neurological efficacy has not been proven, but it does help with depression and insomnia. Reininghaus EZ et al. found that biotin plus placebo treatment for 28 days significantly improved psychiatric symptoms in patients with depression [50]. Besides, vitamin D deficiency can alter the GM, reduce the production of B vitamins in the gut, and affect the quality of sleep [51]. Beydoun et al. through cross-sectional data from the National Health and Nutrition Examination Surveys (NHANES), found that total serum carotenoid concentrations were associated with a higher chance of short sleep duration (i.e., 5–6 h per night) compared to normal sleep time (7–8 h per night) [52]. The above studies suggest that vitamin metabolism may be important for the maintenance of normal brain activities.

Our study suggests that total sleep time is most likely related to the metabolism of Tyrosine and propionate in GM. Similarly, Deng L et al. found that ferulic acid (FA) and feruloylated oligosaccharides (FOs) were effective in alleviating anxiety and depressive behavior in mice and enhancing the biosynthesis of phenylalanine, tyrosine and tryptophan in depressed mice. This study shows that FA and FOs may help prevent anxiety and depression [53]. Liang XQ et al. found that Yang-Xin-Jie-Yu decoction can produce antidepressant effects in the Chronic Unpredictable Mild Stress (CUMS)-induced depression rat model, and its molecular mechanism includes regulating the host's propionic acid metabolism [54].

Terán-Pérez et al. found that the involvement of steroid hormones, in addition to their role in regulating sexual behavior, also has an important impact on the sleep process [55]. Vargas et al. found that it is possible that the wakefulness that occurs in chronic insomnia may be associated with the aberrant occurrence of cortisol pulses at night [56]. The above research results are consistent with the conclusions reached in our experiment. Therefore, the signaling pathways in GM found in

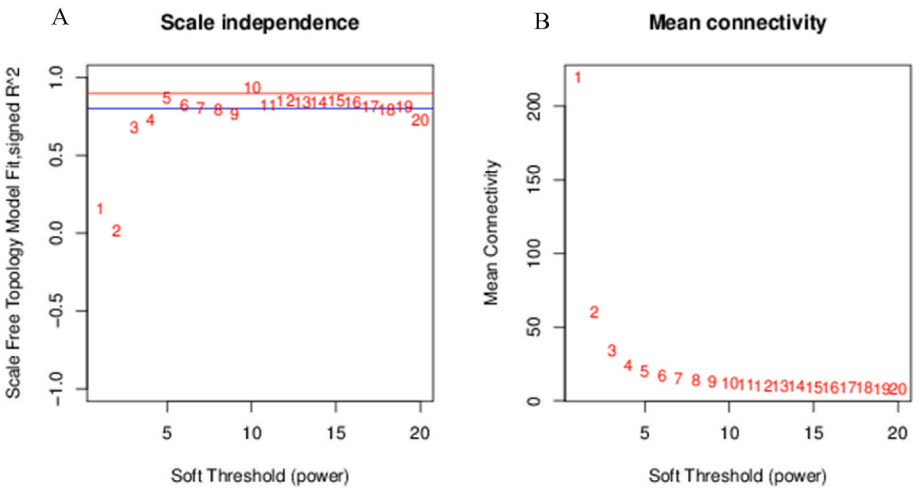

Fig. 6. Analysis of network topology for various soft-thresholding powers: (A) the scale-free fit index as a function of the soft-thresholding power. The x-axis represents the soft-thresholding power and the y-axis represents the scale-free fit index, blue line represent $R^2 = 0.8$; (B) the mean connectivity as a function of the soft-thresholding power. The x-axis stands for the soft-thresholding power and the y-axis stands for the mean connectivity.

Table 4 may affect sleep by influencing the brain. This conclusion may provide clues for further exploration of the specific mechanisms by which GM affects brain activities, as well as the specific mechanisms of MGBA. It is worth mentioning that, according to experimental analysis, Nitrotoluene degradation in GM may be related to sleep efficiency, severe insomnia, total sleep time, and name score in MoCA. This suggests that Nitrotoluene degradation in GM may have a strong association about sleep and insomnia symptoms.

However, this is a cross-sectional study and it is unclear how GM affects symptoms in PI patients. The mechanism of the effect of MGBA in PI patients remains to be studied. Besides, this experiment failed to find a basis for GM's influence on structural changes in the brains of PI patients. The causes of changes in brain structure and function in PI patients need to be further studied.

5. Conslusion

In this study, we explored the relationship between gut microbiota and PI traits by using 16s rRNA sequencing analysis. We found that at the phylum level, the relative abundance of Firmicutes and Actinobacteria in the GM of PIs decreased significantly compared with HCs, while the relative abundance of Bacteroidetes increased significantly. This suggests that the use of Probiotics of Firmicutes and Actinobacteria may improve the symptoms and cognitive function of PI patients.

By perform WGCNA analysis, we found some OTU modules were associated with several important PI marker, including the general conditions, insomnia symptoms, anxiety and depression scale assessments in brains of PI patients. Depending on the PICRUSt2 KEGG pathway enrichment results, we found that.

(1) Insulin signaling pathway, Flavonoid biosynthesis, Ascorbate and aldarate metabolism, Nitrotoluene degradation, Biotin metabolism, and RNA polymerase present in GM may be correlated with sleep efficiency and severe insomnia.
(2) Tyrosine metabolism, Propanoate metabolism, Carbon fixation pathways in prokaryotes, Carotenoid biosynthesis, Systemic lupus erythematosus, Nitrotoluene degradation and Biosynthesis of unsaturated fatty acids present in GM may be correlated with total sleep time and name score in MoCA.
(3) Apoptosis, Steroid hormone biosynthesis, Geraniol degradation, Protein digestion and absorption and Bisphenol degradation present in GM may be correlated with name score in MoCA. In the above three sets of correlation relationships, the correlation decreases sequentially. And we also found that Nitrotoluene degradation present in GM may be correlated with sleep efficiency, severe insomnia, total sleep time, and name score in MoCA.
(4) Chlorocyclohexane and chlorobenzene degradation present in GM may be correlated with sleep efficiency.

Ethics statement

This prospective study has been approved by the Medical Ethics Committee of the Guangdong Second Provincial General Hospital. All subjects consented to participate and signed a written informed consent form.

Declaration of figures authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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CRediT authorship contribution statement

Linghui Nie: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology. **Qian Xiang:** Validation, Visualization, Writing – original draft, Writing – review & editing. **Yaqi Lin:** Methodology, Project administration, Resources. **Yajing Xu:** Methodology, Project administration. **Wanhua Wen:** Investigation, Methodology. **Yingxing Deng:** Investigation, Methodology. **Jingying Chen:** Methodology, Project administration. **Xiqi Zhu:** Investigation, Methodology, Project administration. **Linlin Xie:** Funding acquisition, Investigation, Methodology. **Zhiyong Wu:** Software, Supervision, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial

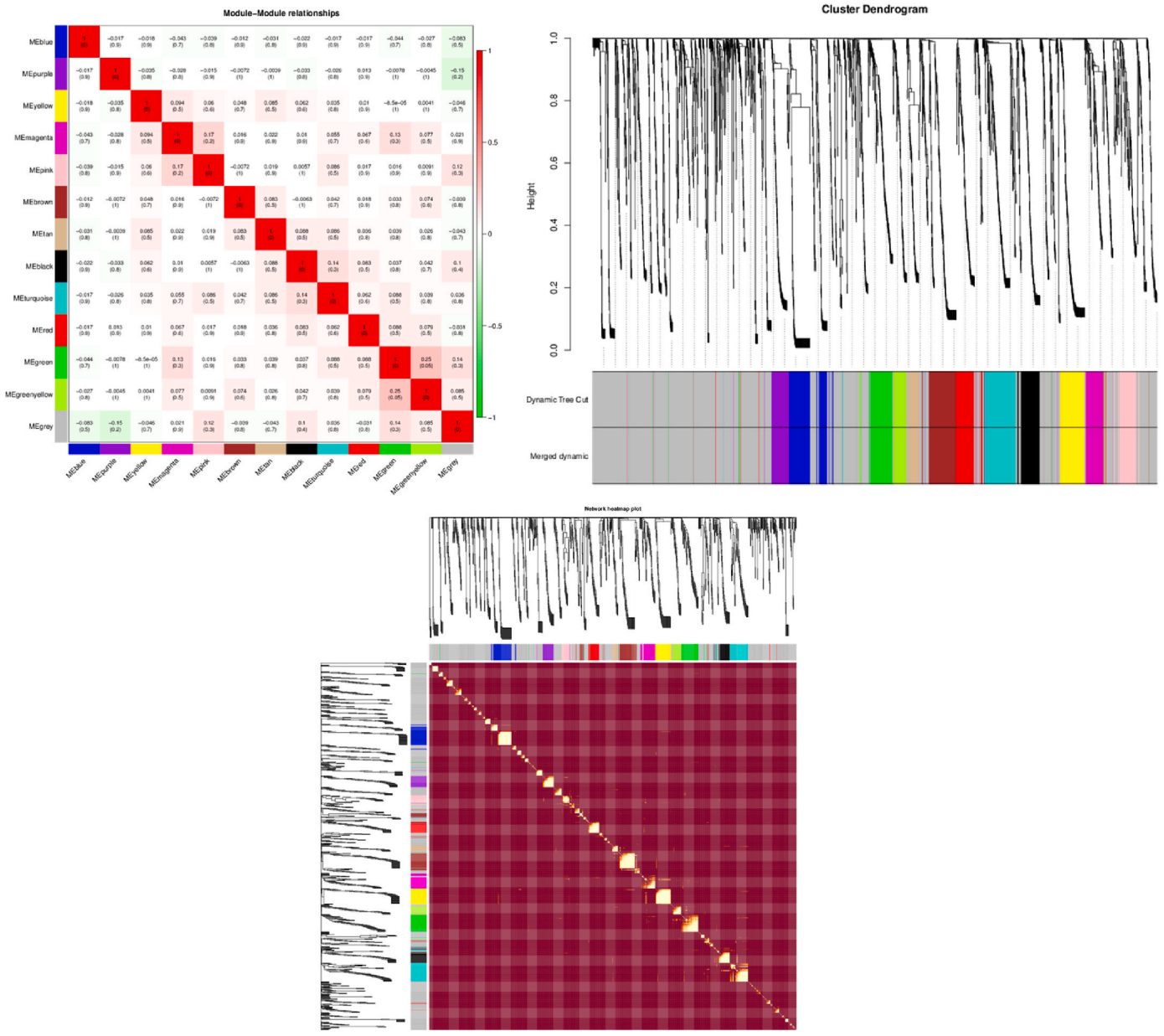

Fig. 7. The modules relationship and distribution. A, Heat map of the eigengene adjacency. The color bars on the left and below indicate the modules for each row or column; B, Cluster dendrogram obtained by average linkage hierarchical clustering. The color below the dendrogram demonstrates the module assignment determined by the dynamic tree cut. 13 modules were depicted in different colors. OTUs didn't classified in any of the color modules were included in the Grey module; C, Heatmap of the topological overlap matrix (TOM) of OTUs. The red color represents a higher overlap.

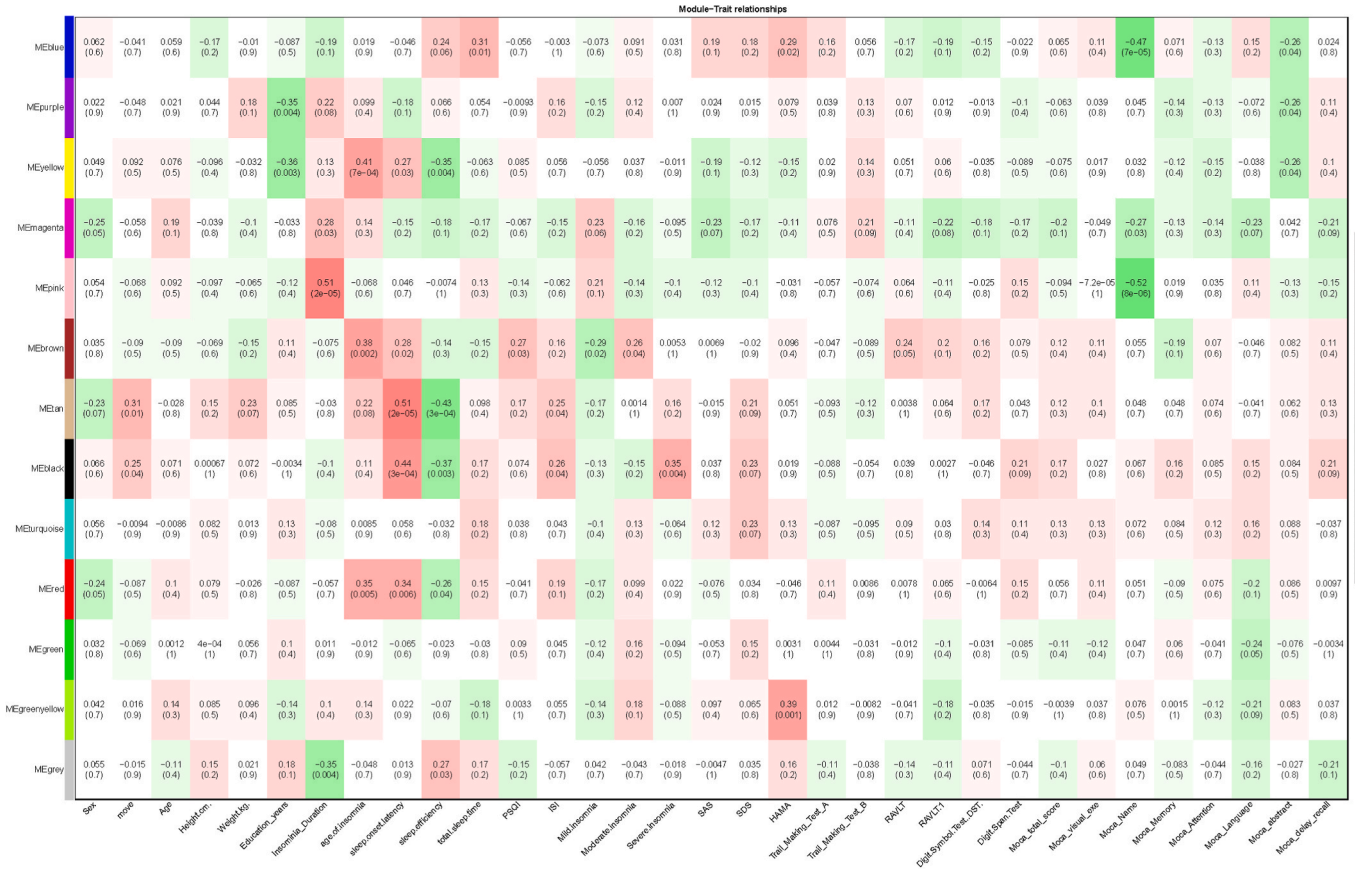

Fig. 8. Module-trait correlation heatmap.

Table 4
The significant enriched KEGG pathways predicted by PICRUSt2.

a. Sleep efficiency with black		
pathway	description	*P*-value
ko04910	Insulin signaling pathway	0.011123030
ko00941	Flavonoid biosynthesis	0.014387815
ko00053	Ascorbate and aldarate metabolism	0.020400892
ko00633	Nitrotoluene degradation	0.023224376
ko00780	Biotin metabolism	0.029072103
ko03020	RNA polymerase	0.029338988
b. Sleep efficiency with yellow		
pathway	description	*P*-value
ko00361	Chlorocyclohexane and chlorobenzene degradation	0.029222996
c. Total sleep time with blue		
pathway	description	*P*-value
ko00350	Tyrosine metabolism	0.015623615
ko00640	Propanoate metabolism	0.015623615
ko00720	Carbon fixation pathways in prokaryotes	0.018520182
ko00906	Carotenoid biosynthesis	0.023183534
ko05322	Systemic lupus erythematosus	0.024503150
ko00633	Nitrotoluene degradation	0.025848474
ko01040	Biosynthesis of unsaturated fatty acids	0.026277917
d. Severe insomnia with black		
pathway	description	*P*-value
ko04910	Insulin signaling pathway	0.011123030
ko00941	Flavonoid biosynthesis	0.014387815
ko00053	Ascorbate and aldarate metabolism	0.020400892
ko00633	Nitrotoluene degradation	0.023224376
ko00780	Biotin metabolism	0.029072103
ko03020	RNA polymerase	0.029338988
e. MoCA Name with blue		
pathway	description	*P*-value
ko00350	Tyrosine metabolism	0.015623615
ko00640	Propanoate metabolism	0.015623615
ko00720	Carbon fixation pathways in prokaryotes	0.018520182
ko00906	Carotenoid biosynthesis	0.023183534
ko05322	Systemic lupus erythematosus	0.024503150
ko00633	Nitrotoluene degradation	0.025848474
ko01040	Biosynthesis of unsaturated fatty acids	0.026277917
f. MoCA Name with pink		
pathway	description	*P*-value
ko04210	Apoptosis	0.019299271
ko00140	Steroid hormone biosynthesis	0.027683277
ko00281	Geraniol degradation	0.028224938
ko04974	Protein digestion and absorption	0.028623568
ko00363	Bisphenol degradation	0.029248469

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101629.

Abbreviations

PI	primary insomnia
HC	healthy control
MGBA	microbiome-gut-brain axis
PSQI	the Pittsburgh Sleep Quality Index
ISI	the Insomnia Severity Index
SDS	Self-Rating Depression Scale
SAS	Self-Rating Anxiety Scale
MoCA	the Montreal Cognitive Assessment
RAVLT	Rey's Auditory Verbal Learning Test

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