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ORIGINAL RESEARCH

Linking Gut Microbiota, Oral Microbiota, and Serum Metabolites in Insomnia Disorder: A Preliminary Study

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Purpose: Despite recent findings suggesting an altered gut microbiota in those suffering from insomnia disorder (ID), research into the gut microbiota, oral microbiota, serum metabolites, and their interactions in patients with ID is sparse.

Patients and Methods: We collected a total of 114 fecal samples, 133 oral cavity samples and 20 serum samples to characterize the gut microbiota, oral microbiota and serum metabolites in a cohort of 76 ID patients (IDs) and 59 well-matched healthy controls (HCs). We assessed the microbiota as potentially biomarkers for ID for ID by 16S rDNA sequencing and elucidated the interactions involving gut microbiota, oral microbiota and serum metabolites in ID in conjunction with untargeted metabolomics.

Results: Gut and oral microbiota of IDs were dysbiotic. Gut and oral microbial biomarkers could be used to differentiate IDs from HCs. Eleven significantly altered serum metabolites, including adenosine, phenol, and phenol sulfate, differed significantly between groups. In multi-omics analyses, adenosine showed a positive correlation with genus_*Lachnospira* (p=0.029) and total sleep time (p=0.016). Additionally, phenol and phenol sulphate had a negative correlation with genus_*Coprococcus* (p=0.0059; p=0.0059) and a positive correlation with Pittsburgh Sleep Quality Index (p=0.006; p=0.006) and Insomnia Severity Index (p=0.021; p=0.021).

Conclusion: Microbiota and serum metabolite changes in IDs are strongly correlated with clinical parameters, implying mechanistic links between altered bacteria, serum metabolites and ID. This study offers novel perspective into the interaction among gut microbiota, oral microbiota, and serum metabolites for ID.

Keywords: insomnia disorder, gut microbiota, oral microbiota, serum metabolites, multi-omics, biomarkers

Introduction

Insomnia disorder (ID) describes a common neuropsychiatric condition caused by a mix of genetic and environmental factors. Its main symptoms include low quality of sleep, trouble falling or maintaining asleep, and reduced performance during the day.[1] About 10% of adults struggle with ID and an additional 20% occasionally experience insomnia symptoms.[2] Currently, the diagnosis of ID relies primarily on subjective symptoms and descriptive criteria based on empirical data, lacking objective diagnostic biomarkers.[3] In clinical practice, polysomnography (PSG) is often necessary to completely exclude other sleep disorders for diagnosis. However, the cost, equipment, and space limitations restrict its widespread implementation. Therefore, a more convenient approach is needed for diagnosing ID. Even while our grasp of the pathogenesis of ID has advanced, a universally acknowledged model has yet to be established.[4] Furthermore, the diagnosis of ID and the selection of treatment options are challenging due to the complexity and diversity of their etiology and the wide range of individual differences. A greater understanding of the pathophysiology of ID may contribute to diagnosing and treating this disease.

Gut microbiota is essential for controlling both the enteric and central nervous system, which impacts functions like sleep and wakefulness, through the microbiota-gut-brain (MGB) axis.[5] Studies on sleep deprivation,[6] sleep restriction[7] and circadian rhythm[8] have shown a link between the intestinal flora and sleep disorders. However, sleep deprivation,

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sleep restriction and circadian rhythm disruption do not fully encompass true ID. There is growing evidence suggesting a strong link between gut microbiota and ID.[9–12] As of yet, how the intestinal flora contributes to ID is uncertain. Furthermore, it is widely recognized that oral microbial dysbiosis not only contributes to systemic diseases[13] but also to neuropsychiatric diseases.[14,15] Nevertheless, there is a scarcity of research on the oral microbiome in ID patients (IDs).

Sleep is connected to the body's metabolism. IDs exhibit metabolic disturbances, such as increased levels of amino acids and glucose accumulation during nighttime.[16,17] Additionally, ID disrupts the diurnal fluctuations of specific metabolites.[18] These studies display that metabolic dysregulation is implicated in the pathophysiology of ID. To date, most research carried out on healthy controls (HCs) have involved sleep restriction or sleep deprivation. There are limited studies specifically dedicated to investigating the metabolomics of ID. Moreover, the human microbiota can release relevant metabolites into the bloodstream to influence the development of disease.[19,20] This could constitute a pathogenic mechanism underlying ID.

We postulated that the gut and oral microbiota contribute to the development of ID and can be utilized as extra diagnostic tools. Furthermore, interplays between the microbiota and serum metabolites may have a role in the emergence of ID. We characterized the gut microbiome, oral microbiome, and metabolites of serum in a cohort of 76 IDs and 59 hCs applying 16S rDNA sequencing and untargeted metabolomics in order to evaluate these assumptions. Furthermore, we conducted a thorough study that integrated various datasets of the gut microbiota, oral microbiota, serum metabolites, and host characteristics to reveal the links between them.

Materials and Methods

Participants

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University (Guangzhou, China) (KY-2019-046). Before the study, all participants gave their consent after being informed. The Mini-International Neuropsychiatric Interview,[21] a psychiatric screening tool, was utilized to identify pre-existing disorders. Two independent psychiatrist diagnosed the patients with ID using the criteria outlined in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5).[22] To participate in the study, subjects experiencing insomnia symptoms required to meet the following criteria: (1) Those aged 18 to 60 years old; (2) Those diagnosed with chronic ID as per the DSM-5; (3) Those without any other mental illnesses as verified by the Mini-International Neuropsychiatric Interview. Through advertisements, well-matched HCs with similar eating habits and geographic location were recruited and screened for psychiatric or physical illness with a semi-structured clinical interview. Participants were excluded based on the following criteria to guarantee uniformity: (1) Those outside the 18 to 60 age bracket; (2) Women who were menstruating, pregnant, or lactating during the experiment; (3) Those diagnosed with mental illnesses or other sleep disorders; (4) Those used medications in the last month; (5) Those regularly took probiotics; (6) Those had used antibiotics in the previous 3 months; (7) Those experienced serious diarrhea or astriction in 3 months; (8) Those suffered from serious oral diseases or oral hygiene problems; (9) Those had not any organic diseases which may affect experiment.

Before enrolling in the study, all individuals completed two nights of PSG, with the initial night dedicated to excluding any additional sleep disturbances. All participants entered the sleep laboratory at 8 p.m. The sleep laboratory environment was kept quiet, comfortable and undisturbed to ensure proper monitoring of temperature, humidity and light, and participants were instructed to schedule their bedtime and wake-up time according to their daily routines. Insomnia symptom severity was evaluated through the use of the Pittsburgh Sleep Quality Index (PSQI) and Insomnia Severity Index (ISI) scales, with daytime drowsiness being assessed using the Epworth Sleepiness Scale (ESS). The Hamilton Anxiety Scale (HAMA) and 17-item Hamilton Depression Scale (HAMD-17) were utilized to assess mood symptoms. We also used a self-administered dietary assessment scale, developed from the Adult Eating Behavior Questionnaire,[23] to assess participants' dietary habits. The supplementary methods provide further details on the collection of data on dietary habits. Finally, 76 IDs and 59 well-matched HCs were recruited from December 2019 to August 2020.

Sample Collection

In total, 267 samples were taken from subjects, including 114 fecal samples (59 IDs and 55 hCs), 133 oral cavity samples (74 IDs and 59 hCs), and 20 serum samples (10 IDs and 10 hCs). The supplementary materials provide a detailed description of the sample collection procedures.

16S rDNA Amplicon Sequencing

Genomic DNA was extracted from the fecal and oral cavity samples using the E.Z.N.A. ®Stool DNA Kit (D4015, Omega, Inc., USA). PCR was performed using optimized primers (341F: 5'-CCTACGGGNGGCWGCAG-3'; 805R: 5'-GACTACHVGGGTATCTAATCC-3') to target the V3-V4 region of the 16S rDNA gene from bacteria. Following DNA amplification, Illumina NovaSeq PE250 platforms were used to sequence the DNA (Illumina, CA, USA). A grand total of 13,360,246 high-grade readings were acquired, averaging 54,090 readings (ranging from 27,552 to 74,614) per sample. By using VSEARCH,[24] all chimera sequences were removed. Using the "Open-Reference" method of QIIME 1.91,[25] sequences without chimeras were processed and classified into operational taxonomic units (OTUs) with 97% similarity. Based on the Greengenes database, taxonomy was assigned using the RDP classifier.[26] The 16S rDNA sequencing data has been stored in the Genome Sequence Archive[27] in National Genomics Data Center,[28] Chinese Academy of Sciences (GSA: CRA014020), which is publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Bioinformatics Analysis

With R package EasyMicroPlot,[29] alpha diversity, beta diversity, principal coordinates analysis (PCoA), and permutation multivariate analysis of variance (PERMANOVA) were conducted. A Manhattan plot was generated by "edgeR", "dplyr" and "ggplot2" to show the difference in OTU abundance between the two groups. For the microbial ecology analysis, the R package "ggClusterNet"[30] was used, along with the model_igraph function and the method "cluster_fast_greedy". False discovery rate (FDR) correction was used for multiple correlation analysis. Spearman confidence index ($abs(r) > 0.6$, FDR-$p < 0.05$) was used to estimate the edges. To identify key signature microbiota, the data at the species level was divided randomly into training (70%) and validation (30%) sets. We created a prediction model with the training set and tested its performance with the verification set. The random forest (RF) algorithm was utilized to calculate importance scores (MDA) and evaluate variable importance in the full RF model. Ten-fold cross-validation using five repeats of the rfcv() function from the R package "randomForest" was used to determine which marker set was the best. This best marker set was subsequently used to develop a more sparse RF model. The "A3" package was used to assess model significance and cross-validated R^2 values using 1000 permutations of the response variable. The significance of each predictor was determined using the "rfPermute" package. A ROC curve was generated using the "pROC" package. Finally, we performed Least absolute shrinkage and selection operator (LASSO) regression analyses on the key taxa screened from the random forests described above, controlling for confounders such as age, sex, BMI, education and dietary habits. This allows us to identify the key taxa most associated with ID. LASSO analysis was performed using "glmnet" package of R.

Metabolome Profiling of Serum Samples

Serum metabolites were identified through analysis with an ultra-performance liquid chromatography-mass spectrometer (UPLC-MS). Serum specimens were evaluated using a TripleTOF 5600 Plus high resolution tandem mass spectrometer (SCIEX, Warrington, UK) in both positive and negative ion modes, followed by chromatographic separation utilizing a UPLC system and a Waters ACQUITY UPLC T3 column (100mm x 2.1mm, 1.8m). Metabolite elution was identified using a TripleTOF 5600 Plus system. LC-MS data were processed using XCMS software 3.2.0 (UC, Berkeley, CA, USA) and R software packages such as CAMERA and metaX toolbox. Retention time (RT) and m/z data were merged to identify each ions by comparing them to in-house and public databases (KEGG and HMDB) to identify each ions. Principal component analysis (PCA) was used to visualize metabolic differences between groups. MetaX was utilized to conduct supervised partial least-squares-discriminant analysis (PLS-DA) to identify key differences between them. Metabolite concentrations were converted to variable importance in the projection (VIP) and fold change ratio for

comparison. The significance of each metabolite was also assessed using independent-samples *t* tests. Differential metabolites that showed statistical significance, had a fold change ratio greater than 1.5 or less than 1/1.5, and had VIP values above 1.0 were selected by matching them to the in-house database.

Multi-Omics Analysis

We collected fecal samples, oral microbiota samples and serum samples from the same cohort of 10 IDs and 10 hCs (20 participants in total) and obtained corresponding microbiome and metabolome data. To establish the association between the microbiota and serum metabolites, we utilized a dual-tier approach. As described previously, a downscaling method was utilized to combine microbiota and metabolite data to perform an advanced correlation study using a modified computational process.[31,32] Then, we applied Spearman correlation analysis to identify correlations among metabolites and bacteria in the low-level correlation analysis. FDR correction was used for multiple correlation analysis. The supplementary methods provide further details on the multi-omics analysis.

Statistical Analysis

Statistical analyses were conducted to assess disparities in clinical demographics data among the study groups. The Student's *t*-test was employed for normally distributed continuous variables, the Wilcoxon rank-sum test for non-normally distributed continuous variables, and either the $\chi 2$ test or Fisher's exact test for categorical variables to compare differences across the groups. Statistical significance was considered as a two-sided $p < 0.05$. R4.2.1 was employed in the statistical analysis.

Results

Clinical Characteristics of Recruited Participants

A summary of this research is presented in Figure 1. After applying the inclusion and exclusion criteria, we enrolled 76 IDs and 59 hCs. Table 1 presented the clinical features of the all subjects. Age, gender, BMI and dietary habit of the two groups were closely aligned. IDs exhibited markedly elevated levels of anxiety and depression, surpassing HCs in scores on both the PSQI and ISI. They also had decreased total sleep time (TST), delayed sleep onset latency (SOL), increased time of wakefulness after sleep onset (WASO_time), reduced sleep efficiency (SE), and decreased rapid eye movement (REM) sleep. The clinical aspects are consistent with the outcomes reported in previous researches.[33,34]

Eventually, we collected 267 samples from the subjects for further analysis (Figure S1). We further categorized all the samples into six groups (FC, feces of the HC group; FI, feces of the ID group; OC, oral cavity of the HC group; OI, oral

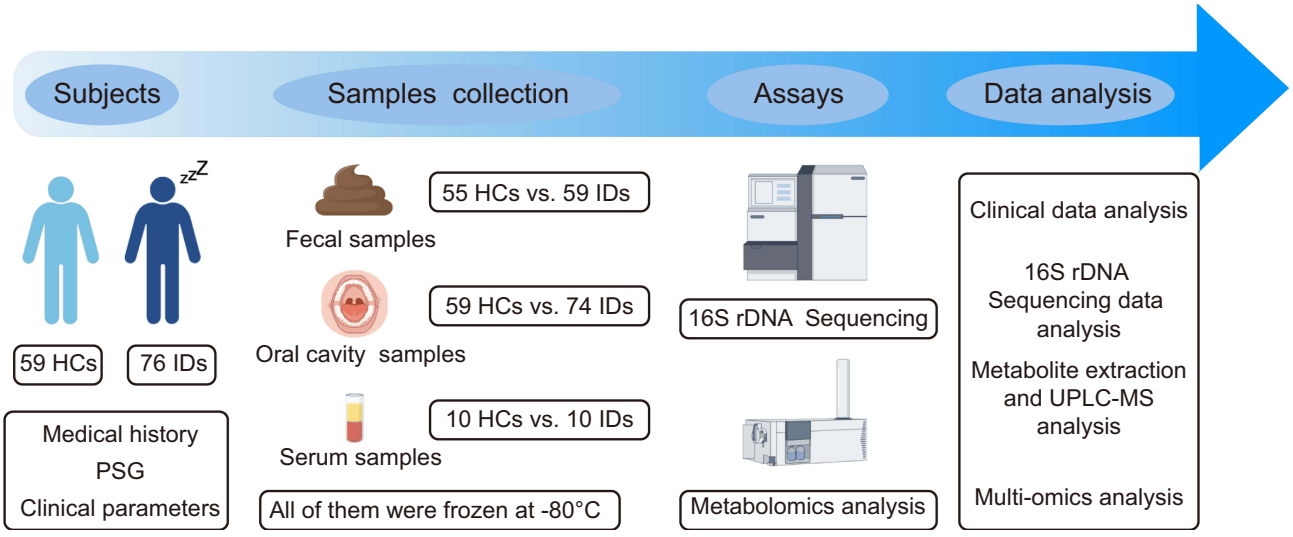

Figure 1 Schematic overview of the study design.
Abbreviations: HCs, healthy controls; IDs, insomnia disorder patients; PSG, polysomnography; UPLC-MS, ultra-performance liquid chromatography-mass spectrometer.

Table 1 Baseline Clinical Characteristics Between All Healthy Controls (59) and ID Patients (76)

Parameters	HC (n=59)	ID (n=76)	*P-value*
Age(years)	29.0 (26.0, 46.5)	30.0 (27.0, 41.8)	0.515
Female, n(%)	35 (59.3%)	47 (61.8%)	0.905
BMI(kg/m^2)	21.4 (± 2.42)	21.0 (± 2.30)	0.291
Education(years)	16.0 (9.25, 18.5)	16.0 (12.0, 17.2)	0.673
Regular diet	10.0 (8.00, 10.0)	10.0 (8.00, 10.0)	0.636
Healthy diet	9.00 (8.00, 10.0)	9.00 (7.00, 10.0)	0.305
Rich diversity in diet	24.0 (23.0, 27.0)	25.0 (23.8, 29.0)	0.136
PSQI	3.00 (2.00, 4.00)	11.0 (9.75, 14.0)	<0.001
ESS	6.00 (3.00, 9.00)	7.00 (3.75, 10.0)	0.254
ISI	2.00 (0, 4.00)	17.0 (14.0, 18.2)	<0.001
HAMA	0.00 (0, 2.00)	6.00 (3.75, 10.0)	<0.001
HAMD	1.00 (0.00, 1.00)	6.00 (4.00, 9.00)	<0.001
WASO_number	25.0 (18.0, 29.5)	24.5 (18.0, 33.5)	0.734
N1(%)	8.80 (6.80, 13.4)	9.70 (6.97, 15.5)	0.373
N2(%)	46.1 (42.8, 48.4)	48.0 (43.6, 54.0)	0.012
N3(%)	21.9 (± 6.91)	20.2 (± 8.07)	0.180
REM(%)	23.0 (19.4, 24.5)	19.2 (16.0, 22.8)	0.001
WASO_time(min)	41.0 (23.8, 59.5)	73.2 (32.1, 99.8)	<0.001
SOL(min)	7.14 (± 5.89)	16.1 (± 17.3)	<0.001
REM_L(min)	84.0 (67.2, 120)	99.5 (68.2, 146)	0.223
TST(min)	417 (378, 466)	374 (346, 410)	<0.001
SE(%)	89.5 (85.6, 93.3)	82.0 (74.6, 88.8)	<0.001
REM_cycle	5.00 (3.00, 6.00)	4.00 (3.00, 5.00)	0.159

Notes: For continuous variables, the means (SD) or medians (IQR) are displayed. Percentages are used to represent categorical variables. The $\chi 2$ test or Fisher's exact test was used for categorical variables, the Student's *t*-test for normal continuous data, and the Wilcoxon rank-sum test for non-normal continuous variables were used to compare the differences between the two groups. Two-tailed statistical significance was considered as $p<0.05$.

Abbreviations: HC, Healthy Control; ID, Insomnia Disorder; BMI, Body Mass Index; PSQI, Pittsburgh Sleep Quality Index; ESS, Epworth Sleepiness Scale; ISI, Insomnia Severity Index; HAMA, Hamilton Anxiety Scale; HAMD, Hamilton Depression Scale; MoCA, Montreal Cognitive Assessment; WASO_number, Number of Wakefulness After Sleep Onset; N1, Non-rapid eye movement stage 1; N2, Non-rapid eye movement stage 2; N3, Non-rapid eye movement stage 3; REM, Rapid Eyes Movement; WASO_time, Time of Wakefulness After Sleep Onset; SOL, Sleep Onset Latency; REM_L, REM Latency; TST, Total Sleep Time; SE, Sleep Efficiency.

cavity of the ID group; SC, serum of the HC group; and SI, serum of the ID group) based on the sources and health conditions. The clinical characteristics of participants with different sample types were shown in Tables S1–S3, respectively. Age and gender, BMI and dietary habit were matched well between the groups. However, PSQI, ISI, TST, REM, SOL, WASO_time, and SE were significantly different.

Gut Microbial Profiling of IDs and HCs

From the 114 fecal samples, 6,232,568 high-quality sequences were used for the analysis. Rarefaction analysis of Richness, Shannon and Good's coverage indices indicated that all the samples were saturated and had sufficient sequencing depth for capturing all bacterial species and further analysis (Figure S2A-C). The Chao1 and ACE indices showed that the diversity of gut microorganisms was significantly larger within the FI versus the FC (Figure 2A). Furthermore, a PCoA analysis was conducted, revealing a notable structural difference between groups based on Bray-Curtis matrices (PERMANOVA tests, $p = 0.038$) (Figure 2B). Based on the Venn diagram, 202 of the 718 OTUs were unique to the FI, whereas 455 were shared (Figure S2D). A Manhattan plot revealed composition discrepancies between groups, with notable deviations in the Firmicutes, Proteobacteria, Actinobacteria, and Acidobacteria phyla (Figure 2C).

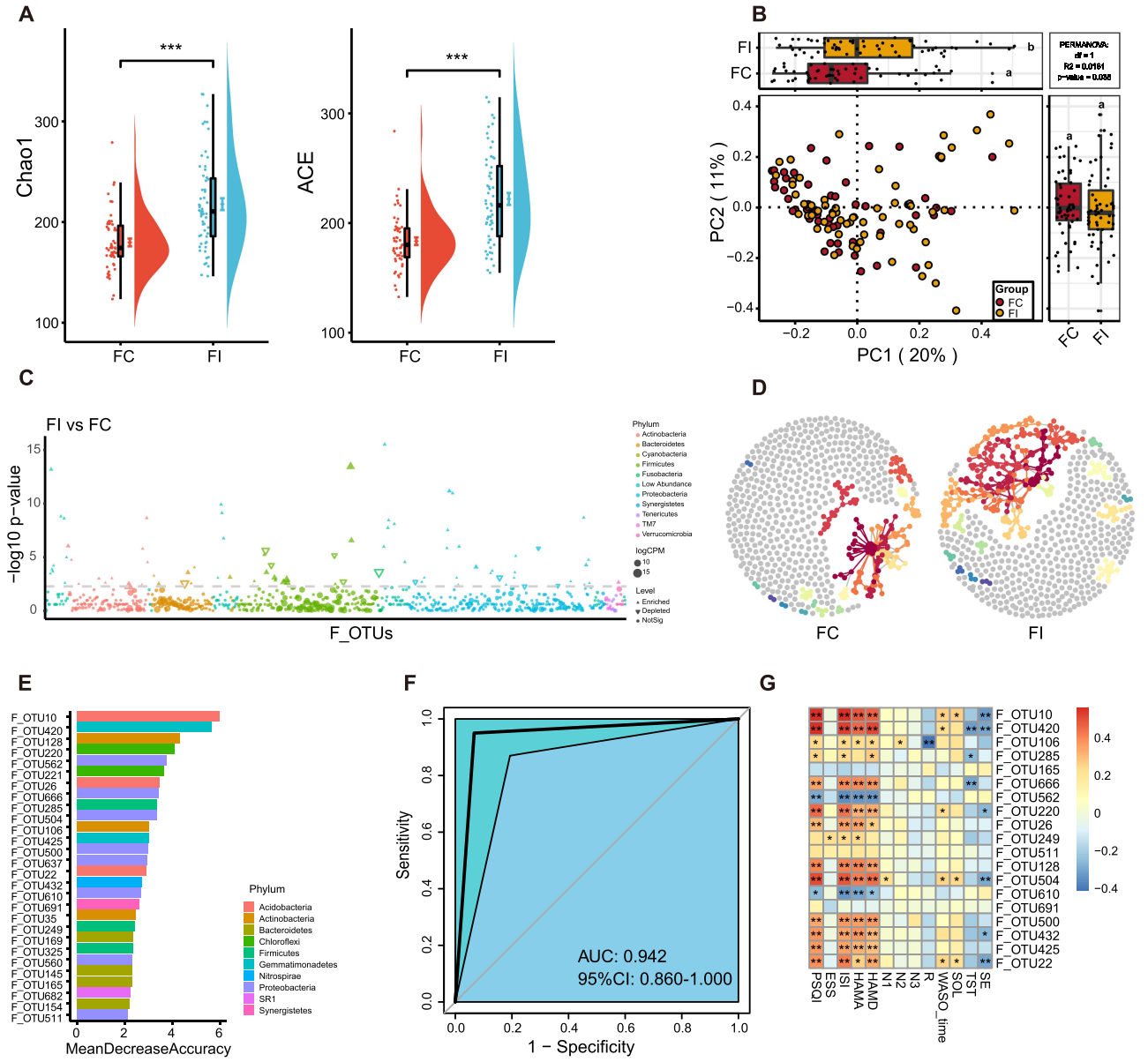

Figure 2 Gut microbial profiling of IDs and HCs. (**A**) As estimated by Chao1 and ACE index, gut microbial diversity was significantly higher in the FI compared to the FC. (**B**) The PCoA based on Bray-Curtis distance showed that the gut taxonomic composition was significantly different between both groups (R^2 = 0.0161, p = 0.038). (**C**) Manhattan plot showed significant structural difference and identified 82 differential OUTs between the two groups. (**D**) Visualization of the constructed networks. The left network was built using the gut microbiota of FC, while the right network was constructed using the gut microbiota of FI. Modules consisting of two or more nodes are depicted in various colors, while the remaining nodes are shown in gray. Nodes with a high degree tended to be cluster together, whereas nodes with a low degree were dispersed throughout the surrounding network. (**E**) The top 28 biomarker bacterial species were identified by applying RF classification of the relative abundance of the gut microbiota in the subjects with fecal samples. Biomarker taxa are ranked in descending order of importance to the accuracy of the model. (**F**) An accurate sparse RF model achieved good diagnostic efficacy with an AUC value of 0.942. (**G**) Heatmap showing the correlation between the key gut OTUs and clinical parameters. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Abbreviations: IDs, insomnia disorder patients; HCs, healthy controls; FC, feces of the healthy control group; FI, feces of insomnia disorder group; PCoA, principal coordinate analysis; RF, random forest; AUC, area under curve; OTUs, operational taxonomic units.

Compared to the FC, there were 71 upregulated OTUs and 11 downregulated OTUs in the FI at the species level (Figure S2E and Table S4). Additionally, network analysis was done to look at the connection between ID and the gut ecology. There was a considerable variance in the intestinal microbial network of the FI and FC (Figure 2D). A radar plot generated by the network analysis, which comprised network topological indices, revealed that ID dramatically affected the intestinal flora's systemic complexity (Figure S2F). Further, we used a diagnostic classifier model to assess the gut microbiome's diagnostic usefulness in ID. Initially, 28 gut OTUs were chosen as the best marker set that could accurately

distinguish the two groups in the training set via five times 10-fold cross-validation in the full RF model (Figure 2E and Figure S2G). Next, we enhanced the accuracy of the sparse RF model using the chosen set of 28 gut OTUs, with the out-of-bag (OOB) error rate decreasing from 22.78% to 10.13%. In the validation cohort, the AUC of the sparse RF model was 0.942 (95% CI: 0.860–1), which could effectively distinguish IDs (Figure 2F). Moreover, we evaluated the significance of these 28 gut OTUs and the sparse RF model. Significant results ($p < 0.05$) were obtained for 19 key gut OTUs and the sparse RF model (Figure S2H). Meanwhile, key gut OTUs were significantly associated with clinical parameters (Figure 2G). Finally, we constructed LASSO regression models to select the best key taxa most associated with ID, controlling for confounders such as age, sex, BMI, education and dietary habits. We found that F_OTU420, F_OTU562, F_OTU26, F_OTU285, F_OTU504, F_OTU106, F_OTU500, F_OTU432, F_OTU610, F_OTU691, F_OTU165 and F_OTU511 were the key taxa.

Oral Microbial Profiling of IDs and HCs

From the 133 oral cavity samples, 7,127,678 high-quality sequences were used for the analysis. Rarefaction analysis of Richness, Shannon and Good's coverage indices indicated that we acquired high-quality data for downstream analysis (Figure S3A–C). A substantial drop in the diversity of oral microorganisms was found within the OI relative to the OC by the Chao1 and ACE indices (Figure 3A). Additionally, a PCoA based on the Jaccard distance revealed noteworthy structural distinctions between groups (PERMANOVA tests, $p = 0.001$) (Figure 3B). As to the Venn diagram, 85 of the 670 OTUs were only detected in the OI, whereas 417 were shared (Figure S3D). Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria phyla were found to vary significantly between groups based on Manhattan plot (Figure 3C). In comparison to the OC, there were 42 upregulated OTUs and 85 downregulated OTUs in the OI (Figure S3E and Table S5). We also conducted a network analysis to examine how ID interacts with the oral ecosystem. The oral microbial network was significantly altered for OI compared with that of OC (Figure 3D). The radar plot computed by the network analysis showed that ID significantly affected the overall complexity of the oral microbial community (Figure S3F). We also assessed the diagnostic capacity of oral flora in ID. At first, 12 oral OTUs were chosen as the best marker set that could accurately distinguish the two groups in the training set via five rounds 10-fold cross-validation in the full RF model (Figure 3E and Figure S3G). Next, we enhanced the precision of the sparse RF model by utilizing the chosen set of 12 oral OTUs and found the OOB estimate of error rate to be 9.68%. The sparse RF model's AUC in the validation cohort was 0.921 (95% CI: 0.837–1), effectively allowing for the separation of IDs (Figure 3F). Furthermore, we evaluated the significance of 12 oral OTUs and the sparse RF model. We found 8 key oral OTUs and the sparse RF model were statistically significant ($p < 0.05$) (Figure S3H). Moreover, key oral OTUs were significantly associated with clinical parameters (Figure 3G). Finally, we constructed LASSO regression models to select the best key taxa most associated with ID, controlling for confounders such as age, sex, BMI, education and dietary habits. We found that O_OTU569, O_OTU321, O_OTU27, O_OTU534, O_OTU610, O_OTU556, O_OTU150 and O_OTU372 were the key taxa.

Associations Between the Microbiota and Metabolomics Contribute to ID

Microorganism-derived metabolites can impact hosts through multiple pathways, with evidence showing that they can enter the bloodstream and significantly affect host physiology and behavior.[33] Therefore, we analyzed the metabolic profiles of serum samples collected from subjects who provided both fecal and oral cavity samples. Then, we looked into how metabolites and microbiota interact in ID.

We acquired high-quality data (Figure S4A–D). The ultimate data revealed that the positive model yielded 17,077 metabolites while the negative model produced 11,313, with 7044 and 5119 of them being annotated, respectively. A total of 600 and 419 metabolites were obtained from the in-house database (Table S6). Multivariate statistical analysis was performed with PCA and PLS-DA models to examine the grouping of various categories in score plots. PCA did not clearly distinguish the SI and the SC (Figure S4E), but the PLS-DA score plot demonstrated distinct clustering based on their metabolic variations (Figure 4A). Additionally, the permutation test for PLS-DA revealed a negative intercept on the Q2 regression line (Figure S4F), confirming the validity of the PLS-DA model. Finally, compared to those in the SC, we discovered 242 upregulated ions and 237 downregulated ions in SI (Figure S4G). These 479 significantly changed ions matched with 11 metabolites from the in-house database. Compared to those in SC, the levels of 7 metabolites, including

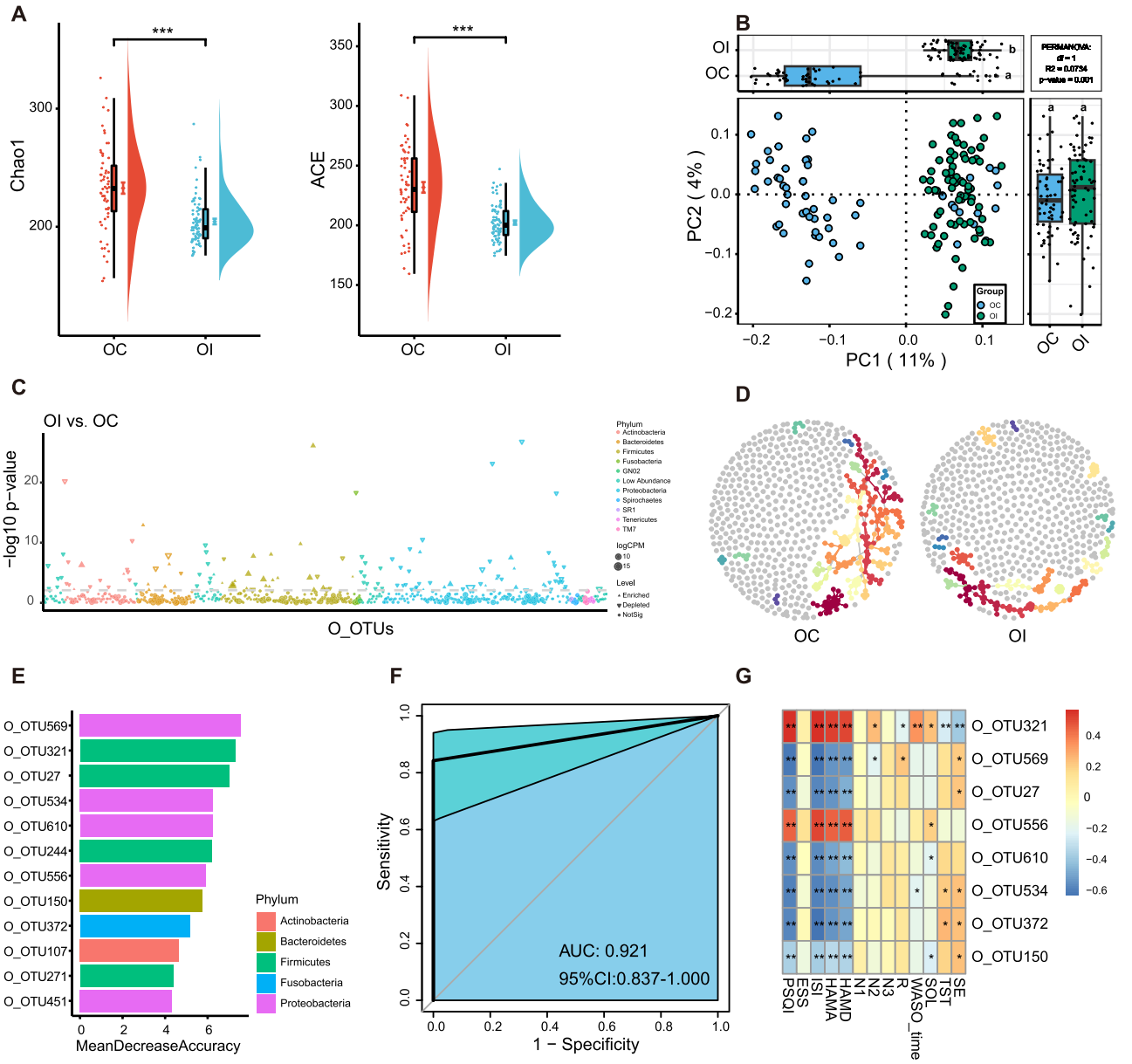

Figure 3 Oral microbial profiling of IDs and HCs. (**A**) As estimated by Chao1 and ACE index, oral microbial diversity was significantly decreased in OI compared with that in the OC. (**B**) The PCoA based on Jaccard distance showed that the oral taxonomic composition was significantly different between two groups (R^2 = 0.0734, p = 0.001). (**C**) Manhattan plot showed significant structural difference and identified 127 differential OUTs between the two groups. (**D**) Visualization of the constructed networks. The left network was built using the oral microbiota of OC, while the right network was constructed using the oral microbiota of OI. Modules consisting of two or more nodes are depicted in various colors, while the remaining nodes are shown in gray. Nodes with a high degree tended to be cluster together, whereas nodes with a low degree were dispersed throughout the surrounding network. (**E**) The top 12 biomarker bacterial species were identified by applying RF classification of the relative abundance of the oral microbiota in the subjects with oral cavity samples. Biomarker taxa are ranked in descending order of importance to the accuracy of the model. (**F**) An accurate sparse RF model achieved good diagnostic efficacy with an AUC value of 0.921. (**G**) Heatmap showing the correlation between the key oral OTUs and clinical parameters. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Abbreviations: IDs, insomnia disorder patients; HCs, healthy controls; OC, oral cavity of the healthy control group; OI, oral cavity of the insomnia disorder group; PCoA, principal coordinate analysis; RF, random forest; AUC, area under curve; OTUs, operational taxonomic units.

phenol, phenyl sulfate and 4-vinylphenol sulfate, were upregulated in the SI. Additionally, 4 downregulated metabolites were observed, with adenosine showing a significant decrease (Figure 4B and Table S7).

To better understand how metabolites and microbiota interplay in ID, we initially performed differential abundance analysis to detect differences in gut microbial communities between groups. Then, we collapsed differential serum metabolites and differential gut microbiota datasets into two clusters each. A summary of the individual bacteria and metabolites within each cluster can be found in Tables S8 and S9. The BF2 gut bacterial cluster and M1 metabolic

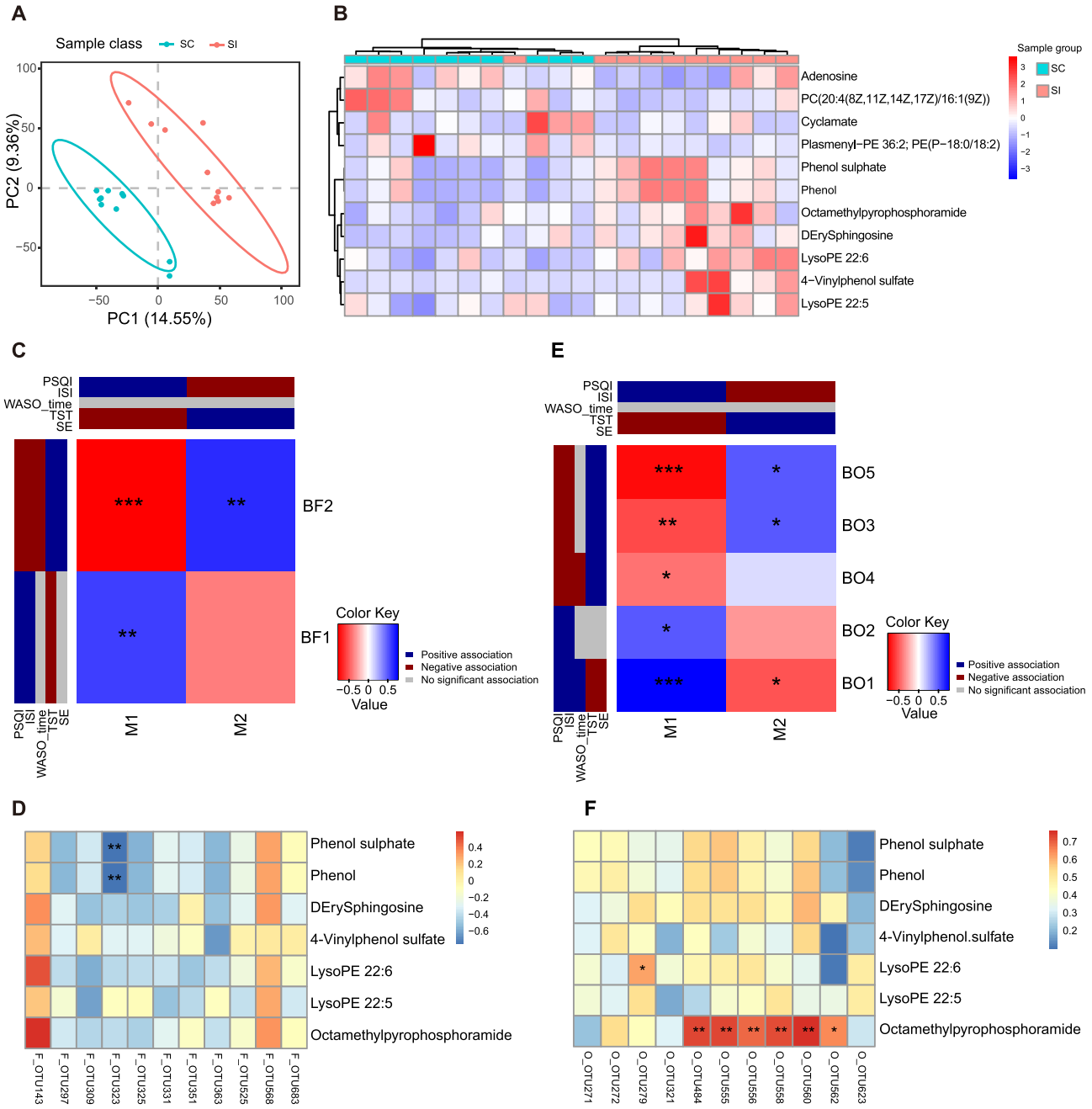

Figure 4 Associations between the microbiota and metabolomics contribute to ID. (**A**) The PLS-DA showed that the serum metabolites in the SI was different from that in the SC. (**B**) The heat map shows the scaled relative intensity of 11 differential metabolites. (**C**) The Spearman correlation coefficient for metabolic clusters-gut bacterial clusters. (**D**) Heatmap showing the Spearman correlation coefficients among 7 distinctive serum metabolites from M1 cluster and 11 distinctive gut OTUs from BF2 cluster. Red color represents positive correlations and blue color represents negative correlations. (**E**) The Spearman correlation coefficient for metabolic clusters-oral bacterial clusters. (**F**) Heatmap showing the Spearman correlation coefficients among 7 distinctive serum metabolites from M1 cluster and 11 distinctive oral OTUs from BO1 clusters. Red color represents positive correlations and blue color represents negative correlations. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.
Abbreviations: ID, insomnia disorder; SC, serum of the healthy control group; SI, serum of insomnia disorder group; PLS-DA, partial least-squares-discriminant analysis; OTUs, operational taxonomic units.

cluster, respectively, showed the strongest correlation with clinical parameters (Table S10). The M1 cluster included phenol and phenol sulphate, both of which were increased with PSQI (r=0.683, FDR-*p*=0.006; r=0.619, FDR-*p*=0.021) and ISI (r=0.675, FDR-*p*=0.006; r=0.602, FDR-*p*=0.021). F_OTU323 (genus_ *Coprococcus*) was part of the BF2 cluster, with a significant negative correlation with PSQI (r=−0.484, FDR-*p*=0.048) but a positive correlation with SE (r=0.550, FDR-*p*=0.045). Following that, the clusters were subjected to cross-domain correlation analysis (Figure 4C). The M1

cluster exhibited an inverse relationship with the BF2 cluster (r = −0.773, FDR-p < 0.001). Ultimately, Spearman correlation analysis was done to see if the gut microbiota in BF2 cluster were linked to metabolites in M1 cluster. We identified several correlations between them (Figure 4D). In this heatmap, F_OTU323 were significantly negatively correlated with phenol (r = −0.747, FDR-p = 0.006) and phenol sulfate (r = −0.747, FDR-p = 0.006). In addition, we discovered that adenosine belonged to the M2 cluster was positively associated with TST (r = 0.677, FDR-p = 0.016), and F_OTU331 (genus_*Lachnospira*) belonged to the BF2 cluster was also positively associated with TST (r = 0.767, FDR-p = 0.002). Since both adenosine and F_OTU331 have a strong positive correlation with TST, we analyzed the correlation between adenosine and F_OTU331, and the results showed that they have a significant positive correlation (r = 0.487, FDR-p = 0.029).

We also obtained the difference of oral microbiota between the two groups. Then, we collapsed differential serum metabolites and differential oral microbiome datasets into 2 and 5 clusters, separately. An overview of the specific bacteria and metabolites within each cluster can be found in Tables S11 and S12. The BO1 oral bacterial cluster and M1 metabolic cluster, separately, showed the strongest correlation with clinical parameters (Table S13). We also found a strong correlation across the BO1 and M1 clusters (r = 0.830, FDR-p <0.001) (Figure 4E). Finally, we identified several correlations between the oral microbiota from the BO1 cluster and metabolites from the M1 cluster (Figure 4F).

Discussion

In this study, we discovered variations in the structure, makeup, and microbial network of the intestinal and oral flora in relation to ID. We also identified specific microbial markers, developed diagnostic models with high efficacy. Furthermore, our study has identified disparities in serum metabolites between the two groups. By integrating multi-omics analysis, we have identified metabolite-bacterium correlation pairs strongly linked to the clinical phenotype of ID.

Sleep is influenced by many factors such as physical and mental health,[35,36] living habits,[37] environmental factors[38] and biological factors.[35,36] There is growing evidence that intestinal flora is strongly linked to sleep disorders.[39–44] In clinical practice, ID is the most common sleep disorder. ID refers to the inability to fall asleep completely, whether in terms of sleeping time or sleeping quality. However, ID are not sleep deprivation, circadian rhythm disorders, sleep apnea, sleep-related movement disorders, and a range of other sleep disorders. In this study, we screened subjects strictly according to the DSM-5 diagnostic criteria. And other sleep disorders were excluded as much as possible by using PSG as a powerful tool. This is beyond the reach of many previous related clinical studies[10–12] focusing on microbiomes of ID.

Previous research has uncovered a dual connection between the intestinal flora and the brain.[45] Studies have shown that gut microbiota is closely linked to the regulation of the sleep-wake cycle.[5] However, how exactly gut microbiota is involved in the development of ID remains unknown. The gut microbiota may be involved in the pathogenesis of ID through bidirectional communication with circadian rhythms,[8] secretion and synthesis of neurotransmitters,[46] and production of inflammatory responses.[47,48] An imbalance in the gut flora may affect the host through the MGB axis, leading to the development of ID. In addition, consistent with previous study,[34] IDs in this study generally had higher levels of anxiety and depression, although the clinical psychiatric assessment excluded co-occurrence of anxiety and depression disorders in the enrolled IDs. However, the anxiety and depression of IDs may be related to their insomnia status, and improving insomnia status may be beneficial in alleviating negative emotions in IDs. In real-world studies, IDs have higher levels of anxiety and depression, and these altered moods may also interact bidirectionally with the gut flora in certain ways.[43] In our previous research,[9] we found clear disparities between IDs and HCs in the structure, function and microbial network of the enteric flora. Additionally, a classifier was developed using two gut microbial markers. The classifier showed excellent precision in identifying ID, as evidenced by an area under the curve (AUC) value of 0.870. Although our previous study identified gut microbiota dysbiosis in IDs, it suffered from small sample size and mismatched baseline data. This study addressed those issues by increasing sample size and controlling for confounding variables such as age, gender, etc., thus improving the comparability and reliability of the results. We discovered that the alpha diversity was greater in IDs than in HCs, which contrasts with the findings of previous studies.[10–12] Physical illnesses can influence the gut microbiota's diversity, which is widely acknowledged. As individuals age, the prevalence of other sleep disorders also progressively rises.[49] In this study, we strictly adhered to the DSM-5 diagnostic criteria for ID and utilized PSG to eliminate the impact of other sleep disorders, resulting in a younger

participant pool. Previous studies solely assessed ID based on subjective experiences and did not fully rule out coexisting conditions with other sleep disorders.[10–12] This study differs from previous studies in some ways may because of these factors. This finding suggests that a more diverse gut microbiota may constitute a compensatory mechanism employed by the body during a specific stage in IDs. Furthermore, this study has developed a more precise diagnostic model than previous studies, with the identified key gut OTUs demonstrating significant correlations with clinical indicators, suggesting a potential relationship between gut microbiota and ID. Clinical assessment of ID could be aided by these findings.

Oral microbiota is not just connected to various oral illnesses[50] but also tied to multiple systemic diseases, significantly affecting human health.[51] We discovered that the alpha diversity was lesser in IDs than in HCs, which contradicts the findings of earlier studies[52,53] that did not rigorously adhere to DSM-5 diagnostic criteria for ID and used PSG to exclude other sleep disorders. Additionally, participants in this study were drug free for one month before enrollment to minimize the drug impact on oral microbiota. Moreover, oral microorganisms exhibit site specificity, and different sampling sites can impact the final results. These factors may explain the discrepancies between this study and previous ones. Moreover, our study has developed a more precise diagnostic model than before, with the identified key oral OTUs demonstrating significant associations with clinical parameters, indicating a potential connection between oral microbiota and ID. Oral sampling is a non-invasive and easily collected method that can provide valuable insights into oral microbial profiles. It complements faecal sampling by targeting a distinct microbial environment that may reflect systemic health conditions or local disease states. It is worth highlighting the simplicity of oral sampling and its use in future disease diagnosis and its complementarity with faecal and serum samples.

Human microbiota contributes to the initiation and course of illness through the release of metabolites into the bloodstream.[19,20] As a result, we examined the link between the serum metabolites and human microbiota. In multi-omics study, we found higher adenosine levels and lower phenol and phenol sulphate levels in HCs compared to IDs. In addition, we also found higher relative abundances of F_OTU331 (genus_*Lachnospira*)[10] and F_OTU323 (genus_*Coprococcus*)[11] in HCs compared to IDs, which is consistent with previous studies.[10,11] Adenosine had an upward trend with F_OTU331 (r= 0.487, FDR-*p*=0.029) and TST (r=0.677, FDR-*p*=0.016). Both phenol and phenol sulfate exhibited a negative correlation with F_OTU323(r=−0.747, FDR-*p*=0.0059; r=−0747, FDR-*p*=0.0059) but a positive correlation with PSQI (r=0.683, FDR-*p*=0.006; r=0.675, FDR-*p*=0.006) and ISI (r=0.618, FDR-*p*=0.021; r=0.603, FDR-*p*=0.021). Our preliminary study showed that the order_*Clostridiales* is a key OTU in the HCs.[9] In this study, we delved deeper into this finding. Gram-positive fermenting bacteria, F_OTU331 (genus_*Lachnospira*) and F_OTU323 (genus_*Coprococcus*), both of which are classified into the order_*Clostridiales* are linked to intestinal health and create short-chain fatty acids that are advantageous.[54] Regulating the intestinal flora and boosting the quantity of short-chain fatty acids may improve sleep quality.[55]

A decrease in F_OTU331 may cause lower adenosine levels in the serum. The theory of sleep homeostasis[56] suggests that the pressure or need for sleep increases with time spent awake and decreases during sleep. The accumulation of adenosine in the brain during prolonged wakefulness may be the physiological basis for the onset of sleep homeostasis. This decrease in the serum adenosine concentration is linked to increased awakening and a decreased TST, aligning with the "sleep homeostasis" theory. Intriguingly, a study showed that genus_*Lachnospira* can be used as a biomarker of late chronotype.[57] ID and circadian rhythm disorders are not the same disease, so genus_*Lachnospira* is heterogeneous in different sleep disorders and may play different roles in different sleep disorders. This heterogeneity deserves to be explored in the future.

Furthermore, Phenol and phenol sulphate are small molecules produced in the intestine by the gut microbiota.[58] According to Kikuchi et al[59], phenol sulphate can predict early proteinuria in patients with diabetic nephropathy and it induces proteinuria by damaging podocytes in mouse models. Additionally, Hsiao et al[60] reported that 4-ethylphenol sulfate, a derivative of phenol sulphate, might impair neurological function and cause anxious behaviors similar to ASD. We speculate that a decrease in F_OTU323 may result in elevated levels of phenol and phenol sulfate in the bloodstream. These toxic metabolites could impact sleep-related brain regions and overall sleep patterns through specific pathophysiological mechanisms, ultimately contributing to the development of ID. Our results emphasize the need of evaluating the quantities of these chemicals in the body, as they may impact the onset, progression, and prognosis of ID.

In addition, we discovered notable disparities in both oral microbiota and serum metabolites between the two groups, which were also correlated. Among the oral microbiota, O_OTU555, O_OTU556, O_OTU558, O_OTU560, and O_OTU562 belong to the Enterobacteriaceae family and are more abundant in IDs than in HCs. Certain diseases can be triggered or worsened by pathogenic symbiotic bacteria in the oral cavity, particularly those belonging to the Enterobacteriaceae family. Periodontal disease can increase the presence of these bacteria, exacerbating colitis.[61] The oral cavity and gut have a close relationship in terms of microbial communities and immune responses. Maintaining intestinal health requires considering oral health. More research is needed on how the oral microbiota influences the development of ID. Identifying the specific oral bacteria that play a role in ID is crucial.

In this study, due to the inherent limitations of cross-sectional study design, although we found associations between microbiota, metabolites and ID, we were not able to establish a causal relationship between them. Longitudinal studies are needed to clarify the nature of these relationships. Additionally, the sample size for serum metabolomics was small. We know that a small sample size reduces the statistical power of the study, so this study is only intended as a preliminary investigation to explore trends and generate hypotheses for future larger studies. The results of the study should be interpreted with caution. Although the observed trends are valuable, it should be emphasized that they need to be verified in future studies.

Conclusion

In summary, our findings provide initial insights into the potential interactions involving the gut microbiota, oral microbiota, and circulating metabolites in ID. Moreover, gut and oral microbiota may serve as effective biomarkers for the auxiliary diagnosis of ID. However, to explore the implications of these findings and establish their relevance for clinical practice, larger prospective multicenter studies are necessary.

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