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ORIGINAL RESEARCH

Associations of Intermittent Hypoxia Burden with Gut Microbiota Dysbiosis in Adult Patients with Obstructive Sleep Apnea

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Purpose: Clinical studies focusing on the association between the gut microbiota and obstructive sleep apnea (OSA) are limited. This study aimed to explore the relationship between intermittent hypoxia and the composition of gut microbiota in adults by analyzing the differences in the characteristics and functional distribution of gut microbiota between patients with different severities of OSA and healthy individuals.

Patients and Methods: A cohort of 113 individuals from the First Affiliated Hospital of Sun Yat-sen University underwent overnight polysomnography from July 2019 to August 2021. The individuals included 16 healthy controls and 97 patients with OSA, categorized by the apnea-hypopnea index into mild, moderate, and severe groups. Fecal samples were analyzed using high-throughput sequencing of the 16S rRNA V3–V4 region to assess gut microbiota composition and function. Correlation analysis was used to evaluate the association between clinical indicators and microbiota markers.

Results: In patients with OSA, the gut microbiota diversity and the abundance of specific microbes that produce short-chain fatty acids decreased ($P<0.05$). The phyla Verrucomicrobia and Candidatus Saccharibacteria, genera Gemmiger and Faecalibacterium, and the species Gemmiger formicilis exhibited decreasing abundance with increasing OSA severity. Correlation analysis revealed a robust association between the proportion of total sleep time, characterized by nighttime blood oxygen saturation below 90%, and the alterations in the gut microbiota, demonstrating that elevated levels of desaturation are correlated with pronounced microbiota dysbiosis ($P<0.05$).

Conclusion: Compared to the control group, the intermittent hypoxia exhibited by patients with OSA may be related to alterations in the composition and structure of the gut microbiota. Our results demonstrate the importance of monitoring hypoxia indicators in future clinical practice.

Keywords: obstructive sleep apnea, gut microbiota, 16S rRNA, intermittent hypoxia

Introduction

Obstructive sleep apnea (OSA) is the most common sleep-disordered breathing, characterized by repeated collapse of the upper airway and apnea during sleep, causing intermittent hypoxia (IH) and sleep fragmentation (SF). Recent studies have revealed a continuous increase in the global incidence of OSA, reaching one billion individuals.[1] In addition to inducing daytime sleepiness, diminished attention, and reduced quality of life,[2] numerous studies have shown that the hypoxic burden and frequent arousals caused by OSA may contribute to the development of metabolic damage.[3–5] With the rising prevalence of OSA risk factors, such as aging and obesity, the medical and social burden attributable to OSA and its complications is anticipated to escalate in the forthcoming years.

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The specific pathophysiological mechanism of OSA is intricate and multifactorial, with IH and SF identified as the principal pathological processes. The inflammatory and oxidative stress cascades are critical pathological features in this process. OSA induces the release of systemic inflammatory mediators, which results in an enhanced and persistent proinflammatory state that leads to multi-organ system diseases.[6] Recent studies have elucidated the crucial role of gut microbiota in modulating the risk of a variety of chronic diseases in maintaining both intestinal immunity and systemic homeostasis. Under typical physiological conditions, gut microbiota and the host are interdependent and restricted to maintain a dynamic balance. Disruption of this balance plays an important role in the occurrence and development of various non-communicable diseases including cardiovascular and cerebrovascular diseases, obesity, type 2 diabetes, inflammatory bowel disease, asthma and cancer.

Recent studies have found that OSA is closely related to changes in the composition and function of the gut microbiota. In animal models, exposure to IH has been proven to cause significant changes in gut microbiota. Moreno-Indias et al found that IH can lead to hypoxia/reoxygenation cycle events within the gut microbiome. IH-exposed mice showed a higher abundance of Firmicutes and a smaller abundance of Bacteroidetes and Proteobacteria phyla than the controls. The alterations in the microbial community may ultimately lead to the occurrence and progression of adverse cardiovascular events and metabolic disorders.[7,8] There is a lack of clinical studies that focus on the association between the gut microbiota and OSA. In the limited observational study, patients with OSA exhibited differences in gut microbiota composition, particularly a reduction in short-chain fatty acid (SCFA)-producing bacteria, which may be associated with the common metabolic disorders observed in patients with OSA.[9] Additionally, the interplay between OSA and the gut microbiota may also involve the gut-brain axis, offering a novel perspective for understanding cognitive dysfunction associated with OSA. While gut microbiota dysbiosis has been demonstrated in animal models and patients with OSA, how OSA may induce these alterations is far from clear. Intestinal microbiota exhibit varying degrees of tolerance to oxygen, and studies indicate that under conditions of IH, the cyclical fluctuations of the hypoxia/reoxygenation cycle within the intestinal lumen and arterial blood are sensitive to the intraluminal environment. These changes may be detected by the gut microbial community, potentially altering the composition and population of oxygen-sensitive microbiota.[7] Furthermore, IH can increase oxidative stress in the duodenum, upregulate the expression of hypoxia-inducible factor-1α, and attenuate the expression of epithelial tight junction proteins. These modifications may facilitate the translocation of a greater number of microorganisms or their metabolites across the intestinal barrier, thereby affecting the stability of the gut microbiota.[10] Additionally, IH may associated with the metabolic pathways of the host, such as altering the metabolism of bile acids that crucial nutritional substrates for the growth and proliferation of gut microbiota. Consequently, this metabolic perturbation could indirectly modulate the population of the microbial communities.[11]

However, previous findings are primarily from the animal experiments, and the characteristics and determinants of gut microbiota dysbiosis in patients with OSA remain unclear. In this study, we aimed to determine the presence of gut microbiota dysbiosis in individuals with OSA and to investigate whether these alterations are associated with the characteristic IH and SF. We hypothesized that there are associations between the severity of OSA, nocturnal hypoxia, and changes in the gut microbiota. Our objective was to provide novel clinical evidence and identify potential therapeutic targets for the assessment and prevention of OSA and its comorbidities from a microbiological perspective.

Materials and Methods

Patients

From July 2019 to August 2021, a total of 113 participants were recruited. Based on the aims of the study, we developed the following exclusion criteria: age less than 18 years; smokers and drinkers; recent history of gastrointestinal illness or surgery, such as gastric ulcer, gastrointestinal polyps, inflammatory bowel disease, gastrointestinal tumors, or gastrointestinal surgery; use of antibiotics, probiotic preparations, or proton pump inhibitors within the past month; history of acute infection, such as acute respiratory infection, acute gastroenteritis or urinary tract infection within the past two weeks; pregnant or lactating women; or history with a history of psychiatric illness that may affect the informed validity of participation in the study. Information, such as demographic characteristics and health status, was collected using a general questionnaire, and after overnight fasting, blood and stool samples were collected.

This study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. The study complied with the Declaration of Helsinki (Ethics Approval No.: [2019]271), and all subjects signed an informed consent form before information collection and sampling.

Laboratory-Based Polysomnography (PSG)

All subjects underwent overnight (10 p.m. to 7 a.m). PSG (Embla® N7000; Oakville, Ontario, Canada), which included electroencephalogram, bilateral electrooculogram, electrocardiogram, mental and tongue electromyography, chest and abdominal exercises, oronasal airflow, finger pulse oxygen saturation, bilateral leg movements, and body position measurements. According to the diagnostic criteria of the American Academy of Sleep Medicine version 3.0,[12] the apnea hypopnea index (AHI) was calculated as the total number of episodes of apnea (sustained halt to airflow for at least 10 seconds) and hypopnea (reduced airflow ≥10 seconds, oxygen saturation ≥3% or with arousal) divided by total sleep time. AHI<5 events/hour were defined as non-OSA (control group), 5≤AHI<15 for mild OSA, 15≤AHI<30 for moderate OSA, and AHI≥30 for severe OSA.

Anthropometric Data & Medical History

Under the guidance of physicians, the subjects filled in a unified format OSA-specific medical history questionnaire that included basic demographics; OSA-related symptoms; OSA diagnosis and treatment history; previous underlying diseases and related treatment history, such as hypertension, diabetes, lipid metabolism disorders, cardiovascular diseases, and liver and kidney diseases; and personal history, including smoking, drinking, and eating habits. Body mass index (BMI) was calculated by dividing weight by the square of height (kg/m^2).

Systemic Inflammatory Cytokines Analysis

Serum samples from the fasting blood were collected in the early morning. The concentration of C-reactive protein (CRP) and inflammatory factors was determined by an automated biochemical analyzer. The concentration of interleukin-1β (IL-1β) in the plasma was determined using a human enzyme-linked immunosorbent assay (ELISA) kit (Mibio, Shanghai, China), according to the manufacturer's instructions.

Sampling, DNA Extraction, and 16S rRNA Sequencing

Biological samples and anthropometric data were obtained without medical treatment. After collection, the blood samples were maintained at 24 °C for 30 min and then centrifuged using a Multifuge X3R centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 3000 rpm for 20 min to obtain serum. Fresh fecal samples, morning urine, and serum samples were immediately frozen on dry ice after collection and stored at −80 °C until further analysis.

Total intestinal microbial DNA was extracted using the cetyltrimethylammonium bromide method. The quality of the DNA was determined by agarose gel electrophoresis, and the sample concentration was accurately quantified using the Qubit Fluorometer and Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The V3 and V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using isolated fecal DNA as a template. PCR amplification of the V3 and V4 regions (forward primer, 5′-ACTCCTACGGGAGGCAGCAG-3′; Reverse primer, 5′-GGACTACHVGGGTWTCTAAT-3′). The fragment range and concentration of the library was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The PCR amplification products are purified using Agencourt AMPure XP magnetic beads and then dissolved in Elution Buffer to complete the library construction. Libraries that met the required criteria were selected for sequencing on the HiSeq platform (Illumina, San Diego, CA, USA) based on the insert size. Sequence assembly is performed using the software FLASH (Fast Length Adjustment of Short reads, version 1.2.11). The resulting data was filtered offline, the remaining high-quality clean data was used for post-analysis, and the reads spliced into Tags through the overlapping relationship between reads. UPARSE was used to cluster Tags under 97% similarity to obtain representative sequences of operational taxonomic units (OTUs). The chimera sequences were compared to the Gold database and UCHIME was used for detection. OTU representative sequences were classified using the Ribosomal Database Project classifier with a minimum confidence threshold of 0.6 and trained on the Greengenes database using Quantitative Insights into

Microbial Ecology (QIIME) for species annotation. The sample species complexity analysis, the species difference analysis between groups, the association analysis and model prediction were performed based on the OTU and annotation results.

Bioinformatics and Statistical Analysis

Based on the QIIME bioinformatics pipeline, the OTU method was used for classification and allocation. The sequencing data from the 113 samples were used to classify and analyze the fecal microbiota, and the Venn diagram was plotted. According to the OTU clustering results, the representative sequences of each OTU were annotated to obtain the corresponding species information and species abundance distribution. Calculate α-diversity indices for each sample, such as Sobs, Ace, Chao1, Shannon, and Simpson, to evaluate the richness and evenness of the microbial communities within the samples. To further explore the differences of community structure among different groups, statistical analysis methods such as *T*-test and linear discriminant analysis effect size were used to analyze the species composition and community results of each group.

The 16S rRNA sequencing data were compared using PICRUSt software to obtain species composition information and analyze the functional differences between different groups. The Kyoto Encyclopedia of Genes and Genomes (KEGG) terms corresponding to the standard OTU was used to calculate enrichment, predict the signaling pathway information, calculate the abundance of different functional classifications, and perform differential analysis. SparCC (Sparse Correlations for Compositional data) algorithm is employed to infer the interspecies correlations within the gut microbiome.

Basic data were categorized into continuous and categorical variables. Continuous variables which followed a normal distribution were characterized by using the mean ± standard deviation. To investigate the status of OSA and its correlation with gut microbiota indicators across varying severities, patients were categorized based on OSA status and severity. Non-parametric tests were used to assess the indicators that did not conform to a normal distribution among groups. Subsequently, one-way analysis of variance, Kruskal–Wallis H, and Chi-square tests were used to compare baseline characteristics among subgroups, and linear-by-linear association assessed the trend of microbiota indicators in response to changes in OSA severity.

Gut microbiota indicators were designated as the dependent variable, whereas the presence and severity of OSA were treated as independent variables. Covariates, such as age and BMI, were adjusted to control for potential confounding factors. Multiple-model linear regression analysis was employed to assess the correlation between OSA status and severity and the composition and function of the gut microbiota while statistically controlling for the influence of confounding variables. In the analysis of the correlation between specific PSG and gut microbiota indicators, a Spearman correlation analysis was conducted both overall and within different subgroups to assess the relationship between variables.

All statistical analyses and graphical representations were conducted using SPSS Statistics version 25 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, Boston, MA, USA). P values of $P<0.05$ was considered statistically significant.

Results

Clinical Characteristics of OSA Patients

After strict inclusion and exclusion criteria were applied, a total of 113 individuals were recruited. The subjects were 39.19 ± 10.70 years old, and their BMI was 25.78 ± 3.81 kg/m^2. PSG evaluation identified 16 healthy controls and 97 patients with OSA, comprising 27, 20, and 50 with mild, moderate, and severe OSA, respectively. Obesity-related indicators in the OSA group, including body weight, BMI, neck circumference, waist circumference, and hip circumference, were significantly higher compared with the healthy controls ($P<0.01$) and showed a progressive increase with the severity of OSA ($P<0.01$). Similar trends were observed for the peripheral blood inflammatory marker CRP. Although the level of IL-1β was significantly elevated in OSA patients compared to healthy controls, it did not exhibit a trend associated with the severity of OSA. In terms of comorbid conditions, systolic and diastolic blood pressures and triglycerides were higher in patients with OSA compared to the healthy controls, whereas levels of high-density lipoprotein were lower ($P<0.05$) (Tables 1 and 2).

Table 1 Characteristics of the Participants with and without OSA

Variables (Mean ± SD)	Total (n = 113)	Non-OSA (n = 16)	OSA (n = 97)	Statistic	P
Sex, n (%)				χ^2=0.68	0.409
Female	17 (15.04)	4 (25.00)	13 (13.40)		
Male	96 (84.96)	12 (75.00)	84 (86.60)		
Age, yrs.	39.19 ± 10.70	33.06 ± 5.86	40.21 ± 10.99	t=−3.88	<0.001
Height, cm	169.37 ± 7.18	169.81 ± 8.59	169.30 ± 6.97	t=0.26	0.792
Weight, kg	74.14 ± 13.47	65.26 ± 12.69	75.61 ± 13.09	t=−2.94	0.004
BMI, kg/m^2	25.78 ± 3.81	22.51 ± 3.32	26.32 ± 3.62	t=−3.93	<0.001
NC, cm	38.83 ± 3.08	36.91 ± 2.34	39.14 ± 3.08	t=−2.77	0.007
WC, cm	93.31 ± 10.16	83.47 ± 9.70	94.93 ± 9.32	t=−4.53	<0.001
HC, cm	100.28 ± 6.51	95.91 ± 5.59	101.00 ± 6.39	t=−3.00	0.003
SBP, mmHg	128.83 ± 16.23	120.00 ± 16.08	130.28 ± 15.86	t=−2.40	0.018
DBP, mmHg	81.57 ± 12.44	77.06 ± 11.98	82.31 ± 12.42	t=−1.57	0.118
FPG, mmol/L	5.26 ± 1.83	4.40 ± 0.47	5.40 ± 1.94	t=−2.05	0.043
TG, mmol/L	1.49 ± 0.99	1.03 ± 0.45	1.56 ± 1.04	t=−2.00	0.048
HDL-C, mmol/L	1.67 ± 0.73	2.24 ± 0.80	1.58 ± 0.67	t=3.55	<0.001
AHI, times/hour	30.38 ± 23.61	2.27 ± 1.19	35.01 ± 22.29	t=−14.34	<0.001
Lowest SPO2, %	77.34 ± 10.90	89.44 ± 3.33	75.34 ± 10.42	t=10.48	<0.001
Mean SPO2, %	93.51 ± 2.55	95.98 ± 1.26	93.10 ± 2.49	t=4.53	<0.001
Maximal-Oxygen Drop, %	16.17 ± 9.00	6.54 ± 2.66	17.76 ± 8.68	t=−10.16	<0.001
T90%, %	0.09 ± 0.13	0.01 ± 0.02	0.10 ± 0.14	t=−5.63	<0.001
TST, h	7.59 ± 1.28	7.75 ± 0.90	7.55 ± 1.36	t=0.55	0.585
CRP, mg/L	1.56 ± 1.03	0.89 ± 0.12	1.67 ± 1.07	t=−2.89	0.005
IL-1β, pg/mL	10.41 ± 7.37	6.87 ± 1.32	10.99 ± 7.79	t=−2.10	0.038

Abbreviations: BMI, Body Mass Index; NC, Neck Circumference; WC, Waist Circumference; H, Hip Circumference; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; FPG, Fasting Plasma Glucose; TG, Triglyceride; HDL-C, High Density Lipoprotein-Cholesterol; AHI, Apnea-Hypopnea Index; T90, Oxygen saturation (SpO2) < 90% of the time; TST, Total Sleep Time; CRP, C-Reactive Protein; IL-1β, Interleukin-1 beta.

Table 2 Characteristics of Participants with Different Severities of OSA

Variables Mean ± SD	Non-OSA (n = 16)	Mild-OSA (n = 27)	Medium-OSA (n = 20)	Severe-OSA (n = 50)	Statistic	P
Sex, n (%)					–	0.292
Female	4 (25.00)	6 (22.22)	2 (10.00)	5 (10.00)		
Male	12 (75.00)	21 (77.78)	18 (90.00)	45 (90.00)		
Age, yrs.	33.06 ± 5.86	37.70 ± 11.59	43.35 ± 9.96	40.30 ± 10.93	F=3.30	0.023
Height, cm	169.81 ± 8.59	170.74 ± 8.16	167.35 ± 8.07	169.30 ± 5.64	F=0.88	0.456
Weight, kg	65.26 ± 12.69	77.57 ± 18.65	71.90 ± 11.16	76.02 ± 9.74	F=3.65	0.015
BMI, kg/m^2	22.51 ± 3.32	26.42 ± 5.00	25.57 ± 2.72	26.56 ± 3.05	F=5.49	0.001
NC, cm	36.91 ± 2.34	38.30 ± 3.17	38.33 ± 2.92	39.93 ± 2.94	F=5.18	0.002
WC, cm	83.47 ± 9.70	94.22 ± 12.04	93.65 ± 8.04	95.83 ± 8.16	F=7.11	<0.001
HC, cm	95.91 ± 5.59	102.76 ± 8.53	99.20 ± 3.96	100.77 ± 5.68	F=4.35	0.006
SBP, mmHg	120.00 ± 16.08	126.93 ± 18.40	128.53 ± 16.41	132.80 ± 13.97	F=2.84	0.041
DBP, mmHg	77.06 ± 11.98	75.61 ± 10.52	83.38 ± 13.58	85.51 ± 11.68	F=5.07	0.003
FPG, mmol/L	4.40 ± 0.47	6.23 ± 3.30	4.92 ± 0.88	5.14 ± 0.85	F=4.34	0.006
TG, mmol/L	1.03 ± 0.45	1.46 ± 0.70	1.40 ± 0.97	1.69 ± 1.20	F=1.89	0.135
HDL-C, mmol/L	2.24 ± 0.80	1.50 ± 0.66	1.80 ± 0.62	1.52 ± 0.69	F=5.16	0.002
AHI, times/hour	2.27 ± 1.19	9.69 ± 3.29	22.41 ± 4.78	53.73 ± 13.54	F=197.06	<0.001
Lowest SPO2, %	89.44 ± 3.33	83.04 ± 4.84	79.75 ± 7.49	69.42 ± 10.10	F=34.38	<0.001

(*Continued*)

Table 2 (Continued).

Variables Mean ± SD	Non-OSA (n = 16)	Mild-OSA (n = 27)	Medium-OSA (n = 20)	Severe-OSA (n = 50)	Statistic	P
Mean SPO2, %	95.98 ± 1.26	94.60 ± 1.24	94.39 ± 1.30	91.77 ± 2.63	F=24.86	<0.001
Maximal-Oxygen Drop, %	6.54 ± 2.66	11.56 ± 4.98	14.65 ± 6.97	22.35 ± 8.31	F=28.81	<0.001
T90%, %	0.01 ± 0.02	0.02 ± 0.04	0.03 ± 0.04	0.18 ± 0.16	F=16.35	<0.001
TST, h	7.75 ± 0.90	6.92 ± 1.42	7.69 ± 1.34	7.88 ± 1.23	F=2.74	0.048
CRP, mg/L	0.89 ± 0.12	1.55 ± 0.23	1.48 ± 0.23	1.81 ± 1.46	F=3.51	0.018
IL-1β, pg/mL	6.87 ± 1.32	10.78 ± 3.39	10.55 ± 6.39	11.28 ± 9.84	F=1.51	0.217

Gut Microbiota Analysis

Altered Overall Structure of the Fecal Microbiota in OSA

A total of 113 fecal microbiota samples were included in this study. The rarefaction curve confirmed that the abundance of the gut microbiota detected by current sequencing was nearly saturated and adequately reflected the composition of the majority of gut microbial communities. The Venn diagram of sample OTU distribution visually represented the similarity and overlap between groups, as well as the similarities and differences in species distribution. A total of 1114 OTUs were identified in patients with OSA compared to 823 in healthy controls, with 343 OTUs unique to the OSA group (Figure 1A). Alpha diversity analysis, including the Sobs, Chao1, and Ace indices, indicated significant differences in gut microbial community structure between the OSA and the healthy control groups, compared to the healthy controls, the indices in the OSA group were

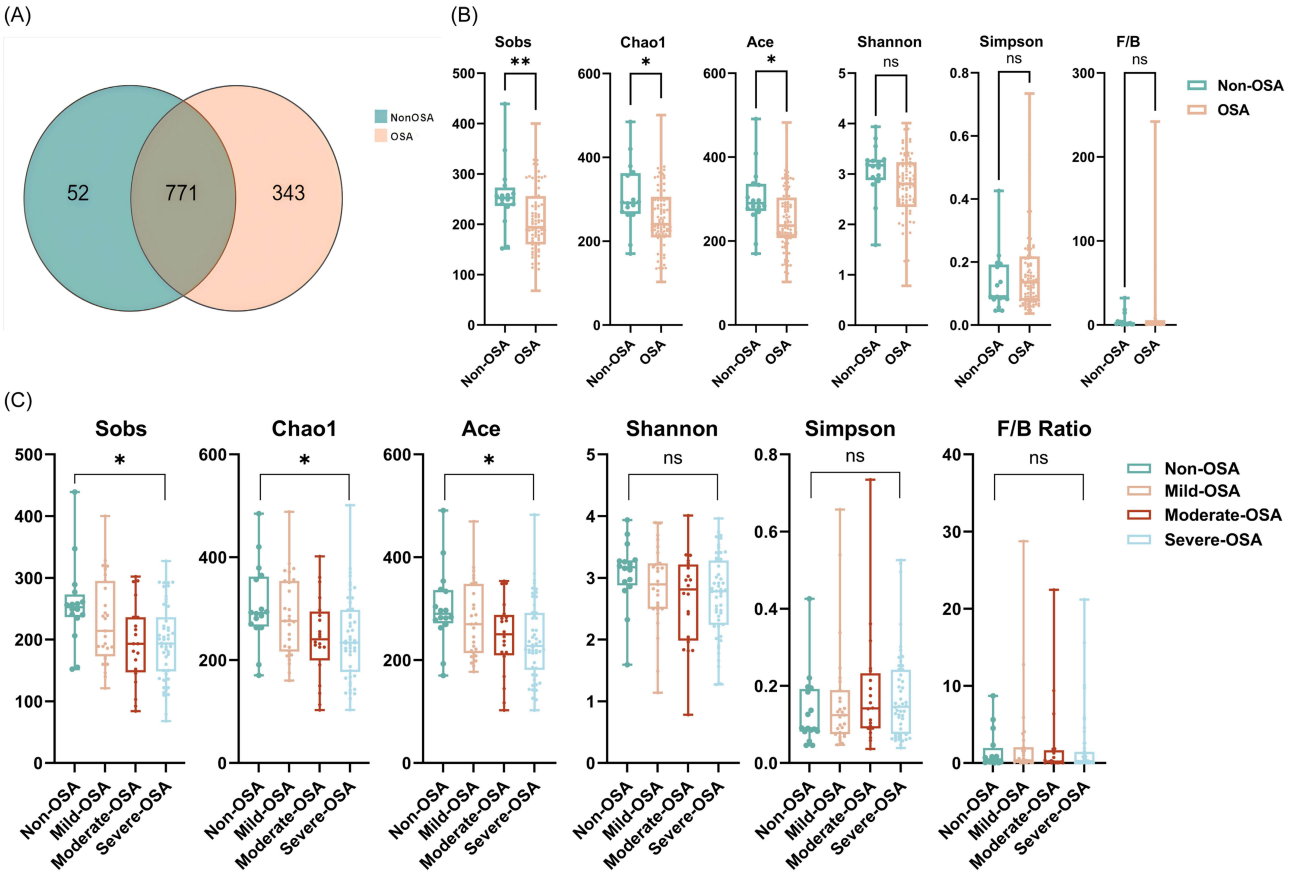

Figure 1 Alpha & beta diversity indices in patients with versus without obstructive sleep apnea. Venn diagram (**A**). Shannon, Simpson and F/B ratio were not significantly changed in obstructive sleep apnea (OSA) patients, while other Alpha diversity parameters including Sobs, Chao1, Ace was reduced in OSA patients (**B**), and decreases with increasing OSA severity (**C**). $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$.

significantly reduced (P<0.01) (Figure 1B). Furthermore, these indices decreased with the severity of OSA (P<0.05) (Figure 1C). By contrast, the Shannon index decreased in the OSA group, and the Simpson index showed an increasing trend which was synchronous with the increase in OSA severity but did not reach statistical significance. Therefore, based on diversity analysis, the overall structure of the gut microbiota in patients with OSA was significantly altered compared to healthy individuals, and there was a decreasing trend in alpha diversity indices, such as Sobs, Chao1, and Ace, with increasing OSA severity (P<0.05).

Composition of Altered Fecal Microbiota in OSA Patients

To evaluate the differences in species composition and relative abundance between the OSA and the control groups, we assessed the composition of species at various levels and analyzed the top 10 key microbial species with the highest relative abundance (Figure 2). Initially, no significant differences were observed in the main microbial communities between the two groups, and both were dominated by Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria, including beneficial bacteria such as lactic acid bacteria and Ruminococcus. Firmicutes was the largest bacterial group, accounting for over 90% of the gut microbiota. A decrease in relative abundance of specific species was observed in some bacteria at the phylum, genus, and species levels in the OSA group compared to the healthy control group (P<0.05). At the phylum level, Verrucomicrobia and Candidatus Saccharibacteria were identified. At the genus level, Gemmiger and Faecalibacterium predominated. The most notable difference at the species level was in Gemmiger formicilis.

The structure of gut microbiota is determined by the dynamic interactions among different microbiota species. We used the SparCC algorithm with FDR adjustment to generate a correlational microbiota interaction network based on the relative abundance of OTUs between groups (Supplementary Figure 1). Compared with the healthy control group, the interaction network of patients with OSA was more complex, and there were more positive and negative correlations between the bacteria. Our data suggest that there may be functional structural changes in the gut microbiota of patients with OSA.

Microbial Functional Dysbiosis in OSA

To determine the metabolic and functional changes in the gut microbiota between patients with OSA and healthy controls, the functional potential of the microbiota was analyzed based on closed reference OTUs using PiCRUSt. A total of 32 level two KEGG pathways were compared, and seven pathways were significantly different between the OSA and control groups. Among them, five were enriched in the control group and two were enriched in the OSA group. Compared with the control group, the signal pathways related to xenobiotics biodegradation metabolism, signal transduction, and environmental adaptation were significantly reduced in patients with OSA (Figure 3).

Intermittent Hypoxia was Associated with Microbiota Dysbiosis

The results described indicated that patients with OSA exhibit dysbiosis in gut microbiota. We conducted further analyses to explore the correlation between indicators from PSG and the bacterial diversity and species abundance (Figure 4). For indicators that reflect the core pathophysiological mechanisms of OSA, such as the AHI, the lowest blood oxygen

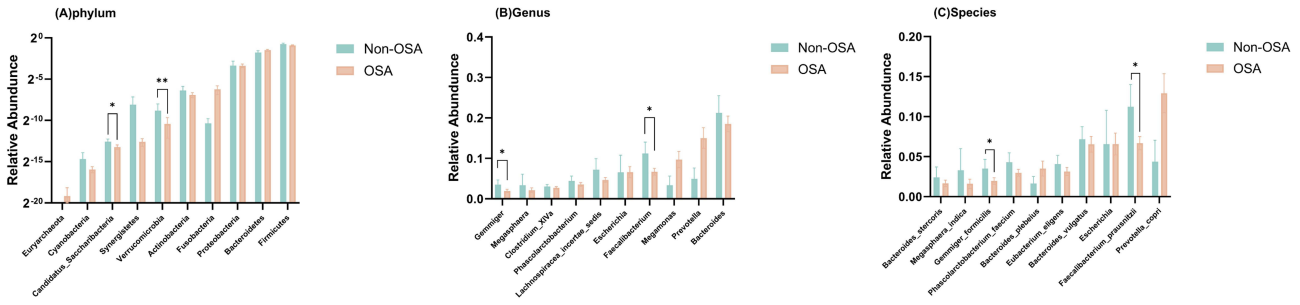

Figure 2 The composition of the fecal microbiome in OSA patients and controls was evaluated at different taxonomic levels, which were manifested by a decrease in the abundance of *Verrucomicrobia* and *Candidatus_Saccharibacteria* at the phylum level (**A**), *Faecalibacterium* and *Gemmiger* at the genus level (**B**), and *Gemmiger_formicilis* abundance at the species level (**C**). ^{ns}P > 0.05, *P < 0.05, **P < 0.01.

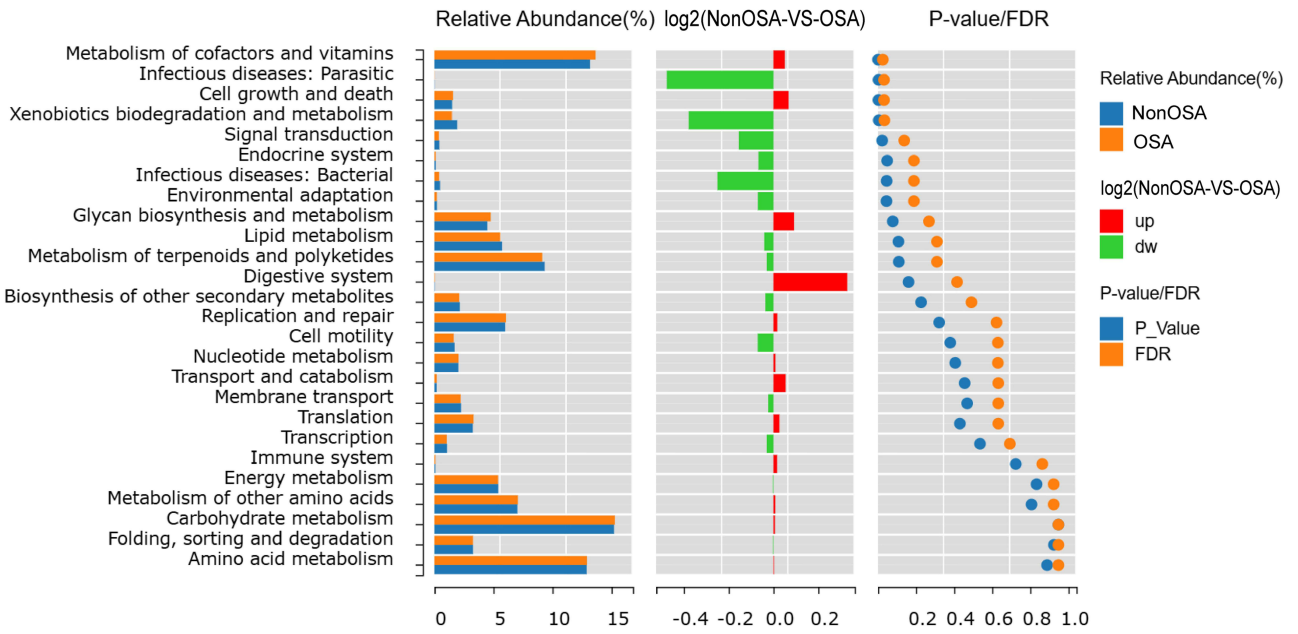

Figure 3 PiCRUSt-based examination of the fecal microbiome of the OSA patients and the healthy controls. The different bacterial functions were evaluated between them based on two-sided Welch's *t*-test. Comparisons between the groups for each KEGG function.

saturation, mean blood oxygen saturation, and the percentage of nighttime with blood oxygen below 90% (T90%), the correlation analysis revealed that although there were differences in the abundance of Verrucomicrobia and Candidatus Saccharibacteria between patients with OSA and healthy controls, these differences did not exhibit significant correlations with other indicators, probably owing to their relatively low proportion in the overall gut microbiota. However, in the abundance of genus and species levels, compared with the AHI, lowest blood oxygen saturation and mean blood oxygen saturation, the T90% index was significantly correlated with the relative abundance of Gemmiger, Faecalibacterium and Gemmiger formicilis ($P<0.05$) (Figure 5).

After controlling for confounding factors, such as age, obesity indicators (BMI, waist circumference), and the presence of metabolic syndrome in a multi-model linear regression analysis, the T90% index remained significantly correlated with indices of bacterial alpha diversity, including Sobs, Chao1, and Ace ($P<0.05$). In contrast to the AHI and other measures of blood oxygen levels, the correlation between T90% and the relative abundance of specific differential microbiota, such as Gemmiger and Gemmiger formicilis, remained significant after adjustment for confounding factors ($P<0.05$). This suggests that, compared to the frequency of sleep fragmentation and extreme values of nocturnal hypoxemia, the duration of intermittent nocturnal hypoxia as a proportion of total sleep time is more strongly associated with a reduction in microbial diversity and the species abundance of differential microbiota ($P<0.05$) (Supplementary Table 1A-1J).

Discussion

OSA is a prevalent sleep-disordered breathing disease, characterized by the recurrent collapse of the upper airway during sleep, such as apnea, hypoventilation, disrupted sleep architecture, snoring, and reductions in oxygen saturation reduction. IH is the primary pathophysiological feature of OSA. OSA is associated with cardiovascular, cerebrovascular, endocrine and other system diseases. Research utilizing extensive clinical databases have found that nearly half of patients with OSA exhibit metabolic impairments, with the risk of metabolic impairment increases with the severity of OSA. Prior studies have indicated that metabolic impairment, driven by hormonal abnormalities and obesity, may exacerbate the progression of OSA. This strongly suggests the bidirectional relationship between metabolic impairment and OSA and the existence of one will promote the development of the other. However, the underlying pathogenesis of OSA-related metabolic impairment remains to be elucidated.

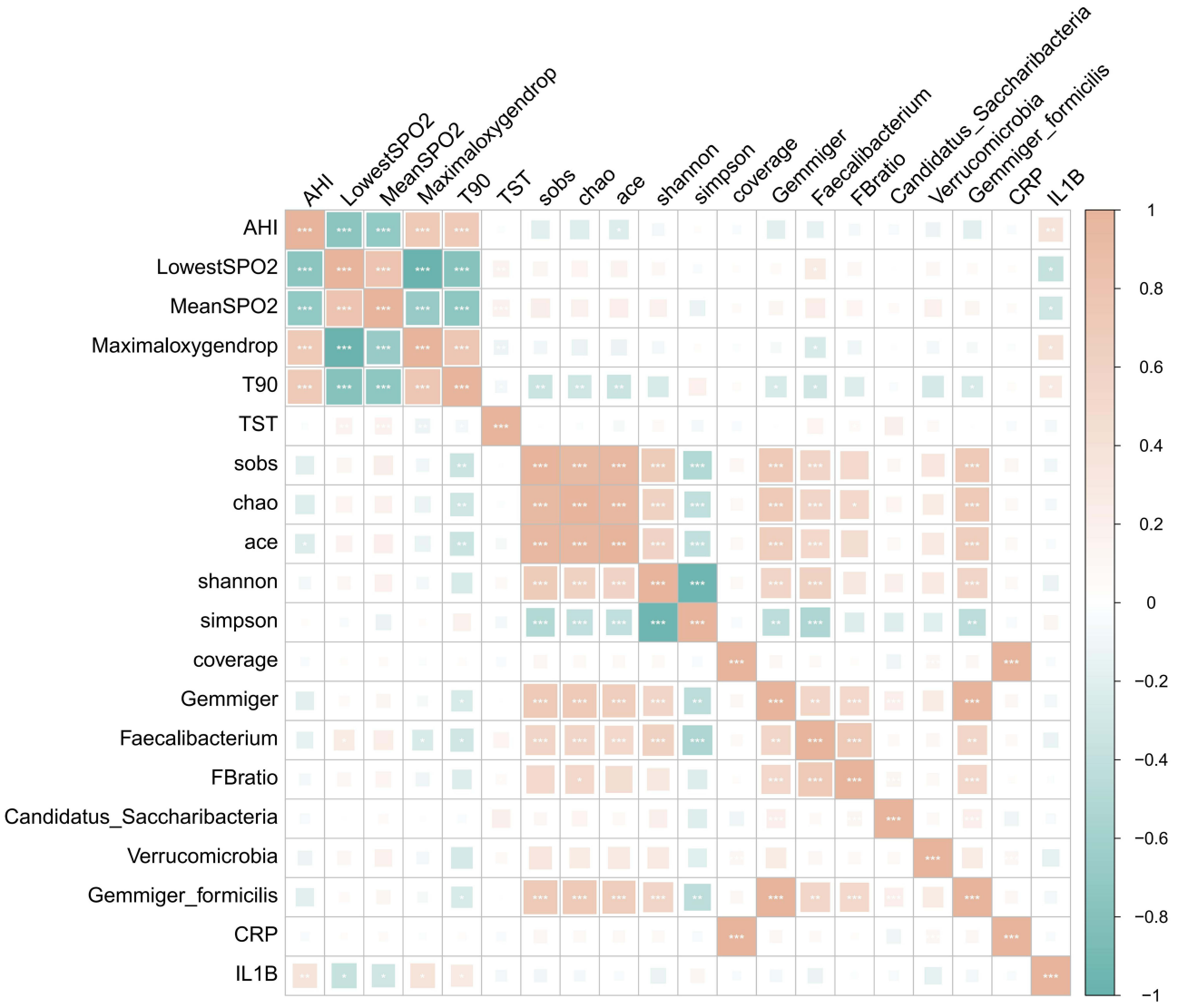

Figure 4 The heatmap shows partial Pearson's correlation coefficients between Differential Microbiota-related Indicators, and Host Immunity, PSG parameters in OSA patients. Pearson's rank correlation (r) and probability (p) were used to evaluate statistic. ^{ns}P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

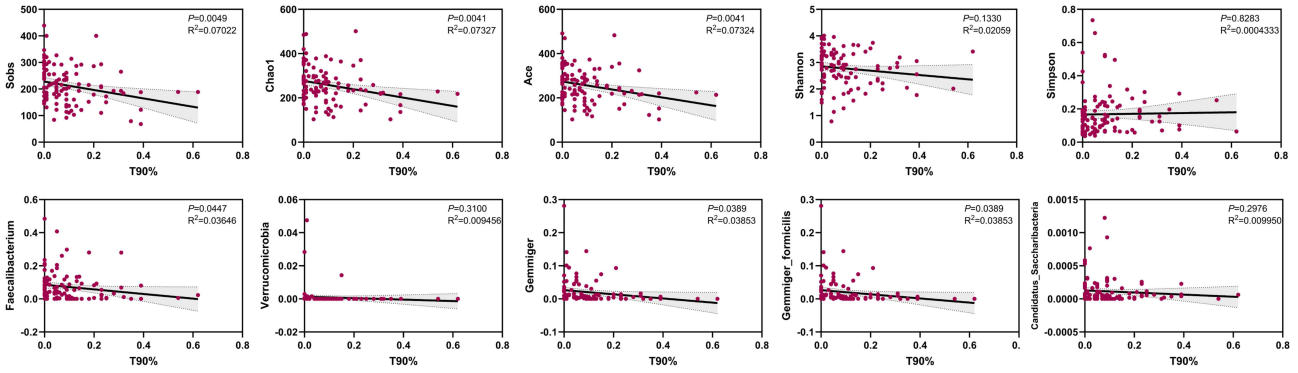

Figure 5 Bivariate linear regression analysis between T90% and indicators of gut microbiota diversity and the abundance of differential microbiota.

Many studies have shown that gut microbiota can maintain intestinal homeostasis and promote human health through various complex mechanisms, therefore, they are often called the "forgotten organ".[13] The analysis of the effect of gut microbiota on diseases has also been extended to other organs and systems. Previous studies using an animal model based on IH, the characteristic pathophysiological mechanism of OSA, have demonstrated different degrees of dysbiosis of gut microbiota, manifested as changes in diversity, abundance and composition structure, suggesting that IH can affect the change of gut microbiota. In recent years, many clinical studies have shown that an imbalance of beneficial and harmful bacteria in the intestinal ecosystem leads to systemic chronic inflammation, which is closely related to the occurrence and development of hypertension and insulin resistance. Therefore, it is reasonable to assume that IH may play an important role in the occurrence and development of OSA-related metabolic damage.

In this study, we conducted 16S rRNA sequencing analysis on fecal samples from patients with OSA and healthy controls. The results revealed significant differences in the composition and specific microbial abundance of the gut microbiota in patients with OSA compared to the controls. There was a significant reduction in alpha diversity indices such as Sobs, Ace, and Chao1, which was significantly correlated with the severity of OSA. The relative abundance of certain microbes at the phylum, genus, and species levels was reduced in the OSA group. At the phylum level, Verrucomicrobia and Candidatus Saccharibacteria were identified. At the genus level, Gemmiger and Faecalibacterium predominated. The most notable difference at the species level was in Gemmiger formicilis. Recent studies have underscored the role of Verrucomicrobia and Gemmiger as pivotal SCFA-producing bacteria.[14] SCFAs are primarily composed of butyrate, propionate, and acetate, and the endogenous concentration of these SCFAs is influenced by a multitude of factors, with intestinal bacterial metabolism emerging as the most significant. SCFAs have been established as a critical link between diet, gut microbiota, and overall health, exerting an influence over the immune system, adipose tissue development, insulin sensitivity, and oxidative stress pathways.[15] They exhibit direct anti-inflammatory properties within the intestinal tract, fostering mucus production, curtailing bacterial translocation, preserving intestinal integrity, and mitigating the host's intestinal inflammatory response.[16]

SCFAs have been identified as potent vasodilators that lower blood pressure in rodent models, modulating the immune response by reducing the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-2, and IL-6, through the histone deacetylase inhibition pathway.[17,18] Monero -Indias et al showed that mice subjected to IH exhibit a reduction in SCFA-producing bacteria and a concomitant rise in mucin-degrading bacteria, precipitating systemic endotoxemia.[19] Ganesh et al further demonstrated that IH can deplete the cecal acetate levels in rats, elevate the mRNA expression of IL-1 and IL-6, and exacerbate inflammatory responses, with the depletion of intestinal SCFAs identified as a pivotal element in these processes.[20] Notably, the transplantation of fecal microbiota from IH-exposed mice into young mice resulted in the emergence of sleep disorders in the recipients, suggesting a potential role for IH-induced alterations in the intestinal microbiota for the etiology of sleep disturbances in patients with OSA.[21] In our study, the observed alterations in Verrucomicrobia, Gemmiger, and Faecalibacterium in the OSA group are of importance as these genera predominantly produce the SCFA butyrate. Butyrate is recognized for its ability to regulate anaerobic conditions in the colon through mitochondrial β-oxidation activation while concurrently suppressing the expression of genes encoding inducible nitric oxide synthase, thereby reducing nitric oxide production and intestinal nitrate levels.

The observed increase in CRP and the pro-inflammatory cytokine IL-1β in the OSA group may reflect a decrease in SCFA levels and an associated rise in peripheral chronic inflammation owing to shifts in the bacterial flora. A diminished SCFA level can compromise the intestinal barrier, leading to increased circulating lipopolysaccharide and the activation of the TLR4-NF-kB inflammatory pathway, thereby promoting a systemic chronic inflammatory state.[22] Our correlation and functional pathway prediction analyses reveal that the OSA group exhibited significant changes in signaling pathways associated with xenobiotic biodegradation metabolism, signal transduction, and environmental adaptation. The dysregulation of these pathways after microbiota disruption may attenuate the body's environmental responsiveness and disrupt normal metabolic and detoxification processes. Consequently, the systemic inflammatory response observed in patients with OSA may stem from the nocturnal hypoxia-induced reduction in SCFA-producing bacteria, allowing metabolites to breach the intestinal barrier and enter systemic circulation, inciting "intestinal endotoxemia" and culminating in chronic metabolic damage associated with long-term OSA.

In our study, we conducted a correlation analysis between the diversity indices of the microbiota, the relative abundance of differential microbiota, and the PSG indices. The results indicated that after adjusting for age, obesity, and certain metabolic disease states, T90% was correlated with both the diversity of the microbiota and the relative abundance of specific bacteria such as Gemmiger, Faecalibacterium, and Gemmiger formicilis. The greater the proportion of time with nighttime hypoxia, the lower the diversity of the microbiota and relative abundance of specific bacteria, particularly those producing SCFAs ($P<0.05$). As a defining feature of OSA, IH has been implicated in altering the gut microbiota through multiple pathways and may induce the metabolic dysregulation associated with OSA. IH has been found to potentially decrease the relative abundance of specific gut microbial populations in mice, such as Bacteroides, Desulfovibrio, and Bifidobacterium, thereby affecting the diversity and functionality of the gut microbiota.[23] This is consistent with the results of our study where the reduced abundances were concentrated among SCFA-producing bacteria in patients with OSA. Furthermore, IH can impact the circadian rhythms of the gut microbiota and metabolome, particularly the levels of bile acids, phospholipids (phosphatidylcholine and phosphatidylethanolamine), and acylcarnitines which may be associated with inflammation and atherosclerosis.[11] IH may increase intestinal permeability, leading to the translocation of gut bacteria or their products across the intestinal epithelium, potentially impacting the host's energy balance and metabolic status, thereby triggering a systemic inflammatory response that could result in metabolic endotoxemia and insulin resistance.[24] A study of rat islet cells found that IH could lead to increased activation of islet B cell apoptosis by up-regulating anti-apoptotic B-cell lymphoma factor 2 (Bcl-2) associated X protein and down-regulating Bcl-2 produced by apoptosis.[25] Taylor et al suggested that IH plays a significant role in insulin resistance by activating the downstream NF-κB pathway and promoting the expression and release of inflammatory factors, including TNF-α and CCL2.[26] Uchiyama et al found that IH could up-regulate the mRNA levels of resistin, TNF-α, and CCL2 in mouse 3T3-L1 and human SW872 adipocytes by down-regulating microRNA-452, leading to the development of insulin resistance. When C57/BL6 mice were exposed to IH, not only the glucose output of hepatocytes was increased but also the expression of various gluconeogenic enzymes in the liver, leading to an increase in fasting blood glucose, impaired glucose tolerance, and accelerating the occurrence and development of T2DM.[27] Another animal study investigated the therapeutic potential of IH and gut microbiota in OSA-related metabolic disorders. Probiotics and prebiotics were shown to enhance the abundance of SCFA-producing bacteria and improve obesity, hepatic steatosis, insulin resistance, and systemic energy expenditure induced by IH.[20]

Previous studies suggest that there is a complex interplay of various mechanisms that may lead to metabolic dysregulation in patients with OSA, such as atherosclerosis and insulin resistance, and IH potentially serves as a significant pathophysiological initiator and accelerator. However, research primarily based on OSA animal models has a limitation in the application of sleep monitoring indicators owing to model constraints. In this study, we conducted a correlation analysis between sleep fragmentation, hypoxia indicators, and the microbiota. We found that compared to sleep fragmentation and the nadir of nocturnal hypoxia, the proportion of time with nighttime oxygen saturation below 90% was significantly correlated with microbiota disruption, indicating that sustained hypoxic conditions may have a higher correlation with microbiota dysbiosis.

Based on the findings of this study, the current use of the AHI and lowest oxygen saturation to assess the severity of OSA and nocturnal hypoxemia may not meet the requirements for refined and multidimensional assessment in diagnosis and treatment. Patients with normal AHI and mild lowest oxygen saturation but who maintain oxygen saturation below 90% for extended periods at night require appropriate attention. It is important to conduct additional examinations, including assessments of the gut microbiota, to identify potential metabolic damage. In terms of treatment, further research into specific mechanisms is needed to guide targeted therapies. However, it may be necessary to explore continuous positive airway pressure treatment modalities and considering the proportion of nocturnal hypoxic time as one of the targets for treatment focus and assessment, aiming to correct hypoxic states and improve microbiota dysbiosis.

Our study has several limitations. Firstly, although we collected dietary habits including eating frequency and types of food during the study, due to the limited number of patients, the quantification of this part of data was inadequate and cannot be analyzed as a confounder. The lack of adjustment of lifestyles including diet may bias the results, however, multiple confounders with well-established associations with both exposure and outcome have been adjusted in the current study to minimize such bias. Secondly, we used the commonly used less time-costly 16S rRNA amplicon sequencing instead of metagenomic sequencing in current study. The 16S rRNA targets characteristic DNA fragments rather than sequencing all DNA segments included in the sample, which may lead to insufficient resolution for species identification below the species level. In our future research, we will also attempt to use metagenomic sequencing. In addition, although this study compared

healthy controls with patients with confirmed OSA and found differences in the abundance of SCFAs-producing bacteria and related factors, due to the cross-sectional design of this study and the lack of direct measurement of SCFAs, it is impossible to infer the specific causal relationship between changes in the microbiota and the disease. In the future, we will conduct in-depth animal experiments to verify the specific mechanisms and carry out clinical multi-omics research to evaluate the therapeutic potential of the microbiota.

Conclusion

In summary, our study reveals that patients with OSA exhibit gut microbiota dysbiosis compared to healthy individuals, marked by a reduction in microbial diversity and a decrease in bacteria that produce SCFAs. We found the nocturnal intermittent hypoxia, particularly the duration of oxygen saturation below 90%, is significantly associated with microbiota dysbiosis, although the causation needs to be confirmed further. Our findings provide novel data for the study of gut microbiota in OSA. It is recommended that clinicians in future clinical practice assess multidimensional information, including the microbiota and hypoxia indicators. Furthermore, whether CPAP therapy can ameliorate OSA and its associated metabolic impairments by restoring microbiota dysbiosis warrants further clinical investigation.

Abbreviations

AHI, apnea hypopnea index; BMI, body mass index; CRP, C-reactive protein; DBP: diastolic blood pressure; ELISA, enzyme-linked immunosorbent assay; FPG: fasting plasma glucose; HDL-C: high density lipoprotein-cholesterol; IH, intermittent hypoxia; KEGG, Kyoto Encyclopedia of Genes and Genomes; IL, interleukin; OSA, obstructive sleep apnea; OTUs, operational taxonomic units; PSG, polysomnography; QIIME, quantitative insights into microbial ecology; SBP: systolic blood pressure; SCFA, short-chain fatty acid; SF, sleep fragmentation; SparCC, sparse correlations for compositional data; TNF-α, tumor necrosis factor-α; TG: triglyceride; TST: total sleep time; T90%: oxygen saturation (SpO2) < 90% of the time.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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